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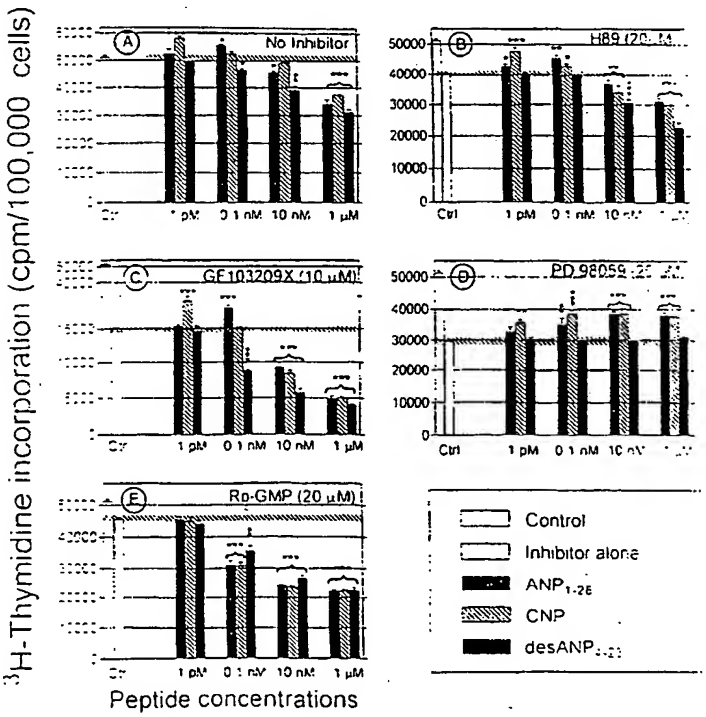
(54) Title: NOVEL NATRIURETIC PEPTIDE RECEPTORS, INTERACTING COMPOUNDS AND METHODS OF REGULATING PROLIFERATION AND/OR SURVIVAL OF NEURONS



(57) Abstract: The present invention provides methods and compositions for regulating proliferation and/or survival of neurons, to treat neuronal death and/or injury, or cancer, and to diagnose neuronal tumors, using compounds such as natriuretic peptides or PHI-related neuropeptides to interact with peptide receptor subtypes on the neurons, and provides a novel receptor on neurons that inhibits proliferation in response to natriuretic and PHI-related neuropeptides, and a novel natriuretic type-C receptor.

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NOVEL NATRIURETIC PEPTIDE RECEPTORS, INTERACTING COMPOUNDS AND METHODS OF REGULATING PROLIFERATION AND/OR SURVIVAL OF NEURONS

This application is based on and claims the priority of U.S. Serial Number 60/151,852, filed September 1, 1999, the contents of which is hereby incorporated by reference in its entirety.

This invention was made with Government support under NIH Grant Nos. HD04612, HD06576 and HD34475. The Government has certain rights in this invention.

FIELD OF THE INVENTION

The present invention relates to compositions for and methods of regulating the proliferation and/or survival of neurons, using compounds, such as natriuretic peptides, that interact with natriuretic peptide receptors on neurons, and to methods of treating neuronal injury or degeneration. The invention also relates to a novel receptor that responds to natriuretic peptides and neuropeptides and to the isolation and characterization of a novel natriuretic peptide type-C receptor.

BACKGROUND OF THE INVENTION

Neuronal injury and neuronal degeneration occur as a result of stroke, brain, retina or spinal cord injuries, ischemia and reperfusion, as well as trauma from surgical procedures, and from other brain disorders and diseases. Diseases such as Huntington's and Alzheimer's, as well as Parkinson's, and amyotrophic lateral sclerosis (ALS), which involve neuronal injury and death, present a serious, ongoing public health challenge to find adequate, cost-effective treatments. In addition, enhanced proliferation and survival of neurons for transplants, such

as brain transplants, is an ongoing goal in medicine.

Neuronal tumors also present a significant, life threatening medical condition, particularly in children. Currently, there are no effective therapies for such diseases. Malignant gliomas are highly aggressive brain tumors that are very resistant to current therapeutic approaches, including surgery, radiation, and chemotherapy. Uncontrolled glial proliferation and vascular invasion are two hallmark characteristics of malignant gliomas. These neoplasms affect approximately 17,000 Americans every year and are invariably fatal. Despite recent advances in the cellular and molecular understanding of cancer, little progress has been made in the treatment outcome of patients with glioblastomas in the past 30 years. There remains no curative therapy for this disease, and the median survival time of affected patients is less than two years. Clearly, the extension of existing knowledge and the development of new, innovative approaches for the treatment of malignant brain tumors are essential.

The presence of peptide receptors on tumor cells has diagnostic as well as therapeutic implications. Peptide receptors have been studied as markers for various cancers (Reubi et al., *Cancer Res.* 57:1377-1386 (1997a)); Reubi et al., *Gut* 42:546-550 (1998); and Reubi et al., *Gastroenterology* 112:1197-1205 (1997b)). Procedures such as receptor scintigraphy may permit non-invasive localization of tumors, as has been shown for somatostatin and vasoactive intestinal peptide (VIP) (Krenning et al., *Nucl. Med. Ann.* 1-50 (1995)). Moreover, where these receptors regulate growth and other functions of tumor cells, their presence or absence may have prognostic implications. Therapeutic applications to inhibit tumor growth include use of peptidase-resistant analogs (Jensen, *Digestion* 58 (Supp. 1): 86-93 (1997)) or radiotherapy using radiolabeled peptides.

Natriuretic peptides include a family of the three structurally related peptide hormones: atrial natriuretic peptide (ANP), brain natriuretic peptide (BNP) and the type-C natriuretic peptide (CNP) (Needleman P. et al., *Ann Rev Pharm Tox*, 29:23-54 (1989); Espiner EA et al., *Endocrinol Metab Clin North Am*, 24(3):481-509 (1995)). Natriuretic peptides are

primarily secreted from the myocardium and neurons. These peptides interact with overlapping specificity with three known receptors (Nakao K, et al., *J Hypertension*, 10: 1111-1114 (1992); Anand-Srivastava MB et al., *Pharmacol Rev*, 45: 455-497 (1994)). ANP binds with high affinity to type A ("NPRA") and C ("NPRC") receptors (Nakao K, et al., *J Hypertension*, 10: 1111-1114 (1992); Anand-Srivastava MB et al., *Pharmacol Rev*, 45: 455-497 (1994)). CNP binds with highest affinity to type B ("NPRB") receptors (Nakao K, et al., *J Hypertension*, 10: 1111-1114 (1992); Anand-Srivastava MB et al., *Pharmacol Rev*, 45: 455-497 (1994)). Des-[Gln^{18}, Ser^{19}, Gly^{20}, Leu^{21}, Gly^{22}]-ANP_{4-23}-NH_2 ($desANP_{4-23}$) is a selective agonist for type C receptor (Maack T. et al., *Science*, 223:675-678 (1987)). Natriuretic peptide receptors differ in their relative affinity for ANP analogues. NPRA binds ANP and BNP with high affinity and CNP with very low affinity. NPRB, on the other hand is relatively selective for CNP. NPRC binds natriuretic peptides with the following affinity profile: ANP>CNP>BNP.

Receptor subtypes A and B (NPRA and NPRB, respectively) contain a single transmembrane domain, and possess guanylate cyclase (GC) activity in their intracellular domain (Pandey KN et al., *J Biol Chem*, 265:12345-12348 (1990); Koller KJ, et al., *Science*, 252: 120-123 (1991)). The type C receptor (NPRC) is similar to types A and B, but lacks the intracellular GC domain (Fuller F. et al., *J Biol Chem*, 263: 9395-9401 (1988)). Despite widespread expression of NPRC, initial studies were unable to identify its signaling pathways.

Because NPRC was found to internalize after binding natriuretic peptides, it was proposed that the receptor might be involved in removing ANP peptides from the circulation (Nussenzveig DR, et al., *J Biol Chem*, 265: 20595-20598 (1990)). It has thus been commonly referred to as the "clearance" receptor. However, more recent studies that have utilized relatively specific ligands for NPRC suggest that it may be positively coupled to phospholipase C (Hirata M et al., *Biochim Biophys Acta*, 1010: 346-351 (1989)) or negatively coupled to adenylate cyclase (reviewed in 4) and MAP kinase pathways (Prins BA et al., *J Biol Chem*, 271, 14156-14162 (1996)). A fourth subtype of natriuretic peptide

receptor (called type D) has been identified in Eel (Kashiwagi M. et al., *Eur J Biochem*, 233:102-109 (1995)). It shows 70% of homology with the clearance receptor and lacks the intracellular GC domain. Expression of the type D receptor in COS cells confirmed its high affinity for $desANP_{4-23}$, but revealed an unexpected sensitivity to HS142-1 (Matsuda Y., *Humana Press Inc, Totowa NJ (Samson WK and Levin ER eds)*, chapt. 17, pp. 289-30712 (1997)), an inhibitor of GC-natriuretic receptor types A and B.

Natriuretic peptides were first revealed to be hormones produced by the heart that regulate vascular tone, sodium and water and other cardiovascular functions through action on the kidney and vascular smooth muscle. Natriuretic peptides regulate central and peripheral body fluid and blood pressure (Goetz K (1988) *Am J Physiol.* 254:E1-15; Brenner BM, et al. (1990). *Physiol Rev*, 70:665-699). All three natriuretic peptides are expressed in the adult brain, where one function appears to involve central control of the cardiovascular system. The peptides have been shown to inhibit the proliferation of several cell types, including vascular smooth muscle cells (Cahill PA et al., *Biochem Biophys Res Comm*, 179: 1606-161313 (1991)) (type C receptors), kidney mesangial cells (Appel RG, *Am J Physiol*, 259: E312-E318 (1990); Haneda M. et al., *Biochem Biophys Res Comm*, 192: 642-648 (1993); Segawa K. et al., *Naunyn-Schmiederberg's Arch Pharmacol*, 357: 70-76 (1998)) (type A and/or B receptors), chondrocytes (Hagiwara H et al., *J Biol Chem*, 269: 10729-10733 (1994)) (type B receptors), osteoblast-like cells (Hagiwara H. et al., *Am J Physiol*, 270, C1311-C1318 (1996)) (type B receptors), and hepatoblastoma cells (Rashed HM. Et al., *Hepalogy*, 17: 677-684 (1993)) (type C receptors). In addition, a report indicated that the type-C receptors mediate inhibition of astrocyte proliferation via inhibition of a MAP kinase pathway (Prins BA. et al., *J Biol Chem*, 271, 14156-14162 (1996)). In contrast to these inhibitory actions, ANP stimulated the proliferation of embryonic cardiomyocytes (Koide M. et al., *Differentiation*, 61: 1-1120 (1996)), and both CNP and BNP were found to stimulate longitudinal bone growth in vitro (Yasoda A. et al., *J. Biol Chem*, 273: 11695-1170021 (1998)) (type B receptors). Moreover, transgenic mice over-expressing BNP and NPRC knockout mice exhibited pronounced skeletal overgrowth (Suda M. et al., *Proc Natl Acad Sci U S A*, 95:2337-42 (1998); Matsukawa N. et al., *Proc.*

Natl Acad Sci USA, 96:7403-7408 (1999)) (type B receptors).

Data suggest that elevation of cGMP may be involved in some of type A and B receptor-mediated proliferative responses (Appel RG, *Am J Physiol*, 259: E312-E318 (1990); Segawa K. et al., *Naunyn-Schmiederberg's Arch Pharmacol*, 357: 70-76 (1998); Hagiwara H. et al., *Am J Physiol*, 270, C1311-C1318 (1996); Koide M. et al., *Differentiation*, 61: 1-11 (1996)), possibly through regulation of the MAP kinase-selective phosphatase MPK-1 (Sugimoto T. et al., *J Biol Chem*, 271: 544-547 (1996)), and/or phosphorylation of PDGF receptor (Awazu M, *Kidney Int*, 52: 356-362 (1997)).

Neuroblastoma cells have been reported to express natriuretic peptides (Niimura S. et al., *Res Comm Chem Pathol Pharmacol*, 63:189-200 26 (1989)) and their receptors (Delporte C. et al., *Eur J Pharmacol*, 207: 81-88 (1991)) (type A or B receptors). They have also been reported to secrete certain endopeptidases (Delporte C. et al., *Eur J Pharmacol*, 227:247-5628 (1992)) that are capable of cleaving natriuretic peptides. Receptors for peptides in the atrial natriuretic peptide (ANP) family appear to be expressed in the proliferative zone of the embryonic brain, indicating a potential role of natriuretic peptides in neurogenesis. Neuroblasts in the embryonic brain and peripheral nervous system, and neuroblastoma tumors also express VIP- and pituitary adenylate cyclase-activating peptide (PACAP)-related neuropeptides as well as natriuretic peptides, and receptors that respond to these peptides. (Lu and DiCicco-Bloom, *PNAS (USA)*, 94:3357-3362 (1997); Nielsen et al., *NeuroReport* 9:2639-2642 (1998)); Sheward et al., *Dev. Brain Res.* 109:245-253 (1998); Waschek et al., *PNAS (USA)* 95:9602-9607 (1998); Muller et al., *J. Biol. Chem.* 264:3647-3650 (1989); O'Dorisio et al., *Reg. Pept.* 37:213-226 (1992); Pence and Shorter, *J. Pediat. Surg.* 27:935-944 (1992); and Vertongen et al., *J. Cell. Physiol.* 167:36-46 (1996)). VIP and PACAP have been shown to regulate the proliferation of both normal cultured embryonic neuroblasts (Waschek et al., *PNAS (USA), supra*; Pincus et al, *Nature* 343:564-567 (1990); and DiCicco-Bloom and Deutsch, *Reg. Pept.* 37:319A (1992)) and neuroblastoma tumor cell lines (O'Dorisio et al., *supra*; Pence et al., *supra*; Wollman et al., *Brain Res.* 624:339-341 (1993); Muller et al., *Mol. Neurobiol*, 10:115-134 (1995); Lelievre

et al., *Neuropeptides* 30:313-322 (1996); and Lelievre et al., *J. Biol. Chem.* 273:19685-19690 (1998)).

Peptide Histidine Isoleucine (PHI), a closely related 27-amino acid neuropeptide encoded on the VIP gene, has been reported to potently inhibit the proliferation of Neuro2a neuroblastoma cells (Lelievre et al., *J. Biol. Chem.* 273:19685-19690 (1998)). Additionally, PHI inhibitory effects have been demonstrated to be selectively sensitive to PD98059, a specific inhibitor of the MAP kinase inhibitor, MEK 1/2 (Alessi et al., *J. Biol. Chem.* 270:27489-27494 (1995). Evidence suggests that VIP and related peptides might coordinate with ANP peptides to regulate certain pharmacological responses through different cyclic nucleotide pathways, and that ANP and VIP might activate a common signaling pathway or share a common receptor (Harmar et al., *Pharmacol. Rev.* 50:265-270 (1998); Torphy et al., *Am. J. Physiol.* 251:G786-G793 (1986); Thirstrup et al., *Eur. J. Physiol.* 319:253-259 (1997); and Akiho et al., *Gastroenterol.* 109:1105-1112 (1995); Murthy et al., *Am. J. Physiol.*, 275:C1409-1416 (1998)).

In addition, receptor autoradiographic studies have provided evidence that high-affinity natriuretic peptide receptors are present in the developing rat brain and associated blood vessels (Zhao L, et al. (1999) *Circulation* 99:605-7; Scott JN, and Jennes L (1991) *Anat Embryol (Berl)* 183:245-9; Zorad S, et al. (1993) *Eur J Pharmacol* 241:195-200; Brown J, and Zuo Z (1995) *Am J Physiol* 269:R261-73). At embryonic day (E) 14, ^{125}I-ANP binding sites were observed over the developing blood vessels and the vascular plexus around the developing brain and in the meningial layer (Scott JN, and Jennes L (1991) *Anat Embryol (Berl)* 183:245-9).

Natriuretic peptides and analogs potently inhibit the proliferation of cultured astrocytes (Prins BA, et al. (1996) *J Biol Chem* 271:14156-62; Levin ER, and HJ Frank (1991) *Am J Physiol* 261:R453-7; Hu RM, and ER Levin (1994) *J Clin Invest* 93:1820-7). This action appears to be mediated by an NPRC-like receptor, and is associated with an inhibition of MAP kinase activity and decreased fibroblast growth factor-2 production. Other groups

have shown that astrocytes also express GC-coupled natriuretic receptors (Sumners C, et al. (1994) *Glia* 11:110-6). One report showed that CNP gene transcripts were produced in astrocytes, indicating the potential for an autocrine/paracrine growth loop for the natriuretic peptide/receptor system in glial cells and associated blood vessels (Yeung VT, et al. (1996) *Neuroreport*, 7:1709-12).

Despite these findings, the biological actions of natriuretic peptides on neurons, including neuronal tumors, and the potential for diagnostic and therapeutic uses of such peptides, have not been elucidated.

There continues to be a need for new diagnostic methods to detect neuronal tumors, and for therapeutic compounds to regulate the proliferation and/or to promote the survival of healthy or injured neurons, as well as for compounds to inhibit growth of neuronal tumors, such as neuroblastomas.

Throughout this application, various publications are referenced within parentheses. The disclosures of these publications are hereby incorporated by reference herein in their entireties.

SUMMARY OF THE INVENTION

The present invention provides methods and compositions for regulating the proliferation and/or prolonging the survival of neuronal cells. The compositions consist of compounds that act on natriuretic peptide receptors, such as Type A, B and C receptors, directly on neurons or indirectly on neurons via Schwann cells, astrocyte, or other glial cell types. In one embodiment of the invention, atrial natriuretic peptides (ANPs) are used to inhibit the proliferation of neurons, for example to treat neuronal tumors. In another embodiment of the invention, ANPs are used to increase the proliferation and/or prolong the survival of neurons, for example to repair injury to nerves or to treat neurodegenerative diseases, to enhance survival of transplanted neurons or to propagate neurons *in vitro*.

The invention further includes methods for treating neuronal injury or degeneration in a subject using the compositions of the invention in an effective dose to increase the proliferation and/or prolong the survival of neurons subject to injury or degeneration.

The methods of the invention include methods of diagnosing neuronal tumors by detecting receptors of natriuretic peptides and/or neuropeptides expressed on neuronal tumors and/or as prognostic or therapeutic indicator in tumor biopsies.

The invention also includes a new receptor, natriuretic peptide neuropeptide receptor or "NNPR", that is expressed on neuronal tumor cells and in the developing brain, and reacts with natriuretic peptides and neuropeptides, and methods of using this receptor.

In addition the invention provides a novel natriuretic peptide type-C receptor that is expressed in Xenopus oocytes and can alter the signaling activity of unrelated receptors.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1A-E are bar graphs depicting the results of thymidine incorporation assays in Neuro2a cells stimulated for 5 hr with increasing concentrations of ANP peptides in the absence (A) and presence (D-E) of signal pathway inhibitors as described in Example I, infra. (A: Control (no inhibitor); B: Protein kinase A inhibitor H89 (20 μM); C: Protein kinase C inhibitor GF109203X (10 μM); D: MEK1/2 inhibitor PD98059 (30 μM); E: Protein kinase G inhibitor Rp-8-pCTP-cGMPS (15 μM). The panel in lower right gives peptide representations. Data were the mean ± SEM of four independent experiments each performed in triplicate. Statistical analysis of the data (ANOVA) were performed (* $p<0.05$, ** $p<0.01$, *** $p< 0.005$)).

Figure 2 is a bar graph depicting the results of thymidine incorporation assays in Neuro2a cells stimulated for 5 hr with increasing concentrations of ANP peptides in the absence or

presence of 20 μg/ml HS142.1 antagonist as described in Example I, infra. (A: ANP; B: CNP; C: $DesANP_{4-23}$. Data were the mean ± SEM of 2 independent experiments each performed in triplicate. Statistical analysis of the data (ANOVA) was performed (* $p<0.05$, ** $p<0.01$, *** $p< 0.005$).

Figure 3 A-C are graphs depicting displacement of different radiotracers (^{125}I: A; ^{125}I-PACAP:B and ^{125}I-PHI:C) by increasing concentrations of native peptides and analogues, as described in Example I, infra. (Binding was performed at 4EC on intact cells. Incubation times were 150, 150 and 180 minutes for the respective radioligands ANP, PACAP and PHI. Symbols for displacing peptides are the same for the three sets of experiments in Figure 3A. Data were the mean ± SEM of three independent experiments, each performed in triplicates. Graphs and curve fittings were computerized using Graphprism software (ISI). Parameters extracted from curves are shown in Table I.)

Figure 4 A-B are graphs depicting displacement of the ^{125}I-ANP radiotracers by increasing concentrations of native peptides and analogues and sensitivity to HS-142-1, as described in Example I, infra. (Binding was performed at 4°C for 150 min on intact cells. A: total ^{125}I-ANP bound in the presence of increasing concentrations of HS-142-1. B: displacement of remaining (HS-142-1 insensitive) ^{125}I-ANP by increasing concentrations of $desANP_{4-23}$. Data were the mean ± SEM of 3 independent experiments each performed in triplicates. Graphs and curve fittings were computerized using ™Graphprism software (ISI). Parameters extracted from curves are shown in Table I, infra).

Figure 5 A-B is a graph showing the dose-dependent effect of natriuretic peptides on intracellular cGMP levels in Neuro-2a cells, as described in Example I, infra. Peptide-induction of the cGMP production in Neuro-2a in the presence of IBMX (10 μM).

Figure 6 A-C are graphs showing the modulation of intracellular calcium concentration in response to neuropeptides and natriuretic peptides in Neuro2a cells as described in Example I, infra. (Cells were growing on glass coverslips; Calcium movements induced by the

indicated peptides were recorded for 250 sec, before addition of the calibration reagents. A: Mobilization of intracellular calcium induced by ANP or by PACAP-27. B: Effects of CNP or PACAP-38 in the same conditions. C: Effects of $desANP_{4-23}$, followed by VIP.)

Figure 7 is copies of photographs of Northern blot analysis of the expression of natriuretic peptide receptors A-ANPR, B-ANPR and C-ANPR in Neuro2a cells, as described in Example I, infra. (Northern blots were prepared as described in Example I. Lanes contained 15 μg of polyA-selected RNA, extracted from Neuro2a, kidney, spleen and 15 μg of total RNA from brain, respectively. Hybridizations were performed overnight at 45°C with the corresponding probes as described in Example I. Ribosomal sub units 28 and 18S were used as size markers.)

Figure 8 A and B is an illustration (8A) depicting polymerase chain reaction (PCR) amplification of NPRC receptor in mouse neuroblastoma cells and in isolated neural tube from E10 embryos, and the resulting autoradiographs (8B), as described in Example I, infra. (Figure 8A: various sets of primers were designed to cover the entire sequence encoding the clearance receptor. Amplified products were run on 2% agarose gel and photographed under UV transillumination after 90 minutes migration at 80V. After Southern transfer, membranes were hybridized with 32P-endlabelled internal oligonucleotides as described in Example I. Figure 8B is autoradiographs of the corresponding PCR reactions, showing specific hybridizations with PCR reaction products from neural tube samples, but none from Neuro2a RNA).

Figure 9 is an illustration summarizing types of receptors for neuropeptides and natriuretic peptides observed in Neuro2a cells as described in Example I, infra, and the putative pathways involved in modulation of neuronal proliferation. (AD: adenylate cyclase; DAG: diacylglycerol; G:G proteins; GC:guanylate cyclase; RTK: tyrosine kinase/receptor).

Figure 10 are bar graphs depicting the thymidine incorporation assays in 4 different neuroblastoma cell lines, as described in Example II, infra. Cells were incubated for 5 hr

with increasing concentrations of natriuretic peptide analogs. Peptide bar codes are given in the first graph. Data were the mean± SEM of four independent experiments each performed in triplicate. Statistical analysis of the data (ANOVA) was performed (* $p<0.05$, ** $p<0.01$, ***$p< 0.005$)

Figure 11 depicts the effects of protein kinase G (PKG) inhibitor on natriuretic peptide-induced modulation of thymidine incorporation in Neuro2a cells, as described in Example II, infra. Data are the mean± SEM of two independent experiments each performed in triplicate. Statistical analysis of the data (ANOVA) has been performed (* $p<0.05$, ** $p<0.01$, ***$p< 0.005$).

Figure 12 illustrates the effects of MAP kinase inhibitor on natriuretic peptide-induced modulation of thymidine incorporation in Neuro2a cells, as described in Example II, infra. Insert gives peptide representations. Data were the mean± SEM of two independent experiments each performed in triplicate. Statistical analysis of the data (ANOVA) was performed (* $p<0.05$, ** $p<0.01$, ***$p< 0.005$).

Figure 13 shows the thymidine incorporation assays in Neuro2a cells stimulated for 5 hr with increasing concentrations of natriuretic peptides in the absence or presence of 20 μg/ml HS142.1 antagonist, as described in Example II, infra. Data were the mean± SEM of 2 independent experiments each performed in triplicate. Statistical analysis of the data (ANOVA) was performed (* $p<0.05$, ** $p<0.01$, ***$p< 0.005$).

Figure 14 illustrates the dose-dependent effect of natriuretic peptides on intracellular cGMP levels in Neuro-2a cells, as described in Example II, infra.

Figure 15 shows the modulation of intracellular calcium concentration in response to neuropeptides and natriuretic peptides in Neuro2a cells, as described in Example II, infra. Cells were grown on glass coverslips; (A: Mobilization of intracellular calcium induced by ANP or by PACAP-27. B: Effects of CNP or PACAP-38 in the same conditions. C:

Effects of desANP$_{4-23}$, following by VIP).

Figure 16 illustrates the comparison of displacement of the ^{125}I-ANP binding by increasing concentrations of native peptides in NG108 and Neuro2a cells, as described in Example II, infra. Parameters extracted from curves are shown in Table II.

Figure 17 shows the displacement of the ^{125}I-ANP radiotracer by increasing concentrations of native peptides and analogues and sensitivity to HS-142-1 as described in Example II, infra. (**A**: two site-binding displacements of ^{125}I-ANP by various peptides in the absence of HS-142-1; **B**: ^{125}I-ANP specifically displaced by increasing concentrations of HS-142-1; **C**: displacement ^{125}I-ANP by increasing concentrations of desANP$_{4-23}$ in Neuro2a cells treated with 25 μg/ml HS-142-1). Data were the mean ± SEM of 2 independent experiments each performed in triplicates. Graphs and curve fittings were computerized using ™Graphprism software (ISI).

Figure 18 depicts Northern blot analysis of natriuretic peptide receptors NPR-A, NPR-B and NPR-C gene expression in Neuro2a cells, as described in Example II, infra.

Figure 19 depicts NPRC gene expression in E10.5 mouse embryos detected by *in situ* hybridization, as described in Example III, infra. All are coronal sections. Dorsal/ventral and anterior/posterior orientations are indicated. Panel D is a dark field view of C (to better visualize the silver grains); all others are bright field photos at different levels of the embryo. 4V = fourth ventricle; PNVP = perineural vascular plexus; CA = carotid artery; R = opening to Rathke's pouch; DI = diencephalon; NC = notochord; A = dorsal aorta; FG= pharyngeal region of the foregut; TV = telencephalic vesicle; 5g= trigeminal ganglia.

Figure 20 illustrates the NPRC gene expression in E12.5 mouse embryos, as described in Example III, infra. Panels A, B, I and J are coronal sections; C and D are in a plane parallel to the spinal cord, G and H are sagittal sections. B, D, F, and J, are dark field

views of A, C, E, and I, respectively. H is a magnification of the area within the rectangle in G. Dorsal/ventral and anterior/posterior orientations are indicated. TV telencephalic vesicle; PNVP = perineural vascular plexus; DRG = dorsal root ganglia; 5n = trigeminal nerve; 5g = trigeminal ganglia; 5max = maxillary nerve; 5 man = mandibular nerve; RP = roof plate; FP = floor plate; LV = lateral ventricle. Arrows in I and J point to one of several capillaries (Ca) shown in this section of the telencephalon.

Figure 21 shows the NPRB gene expression in E10.5 mouse embryos, as described in Example III, infra. A and B are bright and dark field views, respectively, of a coronal section of the hindbrain (dorsal/ventral orientation is indicated). The arrows point to the layer of postmitotic neurons. C is a dark field view in the frontal plane of the spinal cord (anterior/posterior orientation is shown). The bright field view of C is not shown, but is semiadjacent to that shown at different magnification in Figure 2C. 4V = fourth ventricle; 5g = trigeminal ganglia; DRG = dorsal root ganglia.

Figure 22 illustrates the CNP gene expression in E12.5 (A-D) and E14.5 (E-F) mouse embryos. B, D, and E are bright field views of A, C, and E, respectively, as described in Example III, infra. A and B are sagittal sections. C-F are coronal sections. Dorsal/ventral and anterior/posterior orientations are indicated. M = medulla; SC = spinal cord; VC = primordial vertebrate; CC = central canal of spinal cord, 4V = fourth ventricle; 3V = third ventricle. Du = cells associated with the dura. Arrows in C and D point to hybridizing transcripts in the VZ of the dorsal part of the spinal cord.

Figure 23 shows the inhibition of neuroblast proliferation by natriuretic peptides, as described in Example III, infra.

Figure 24 depicts the nucleotide and deduced amino acid sequences of a full-length cDNA encoding a Xenopus type-C natriuretic peptide receptor (NPR-C), as described in Example VI, infra. The black box indicated the location of the putative transmembrane spanning domain. Codons and Cys residues (in Bold) locate the putative inter and intrachains disulfide bounds, whereas grey boxes point out NXT/S sequences implicated in potential N-

glycosylation.

Figure 25 illustrates the alignment of the deduced amino acid sequence of XNPR-C with mammalian and eel sequences of type-C natriuretic peptide receptors, as described in Example VI, infra. Dark gray boxes delineate identities between all the four sequences presented, whereas light gray boxes point out the amino acids specifically conserved between Xenopus and Eel.

Figure 26 shows the alignment of the deduced amino acid sequence of XNPR-C receptor with the Xenopus guanylate cyclases XGC-1 and XGC-2 (Genbank Accession number AB 025111.1 and 025111.2), as described in Example VI, infra. Dark gray boxes delineate the putative transmembrane spanning domain presented, whereas light gray boxes point out the amino acids specifically conserved between XNPR-C and XGC-1 or XGC-2. Black boxes locate cystein residues potentially implicated within disulfide bounds. Amino acids that belong to the natriuretic peptide receptor motif and that are conserved between NPR-C and GC are indicated in bold characters.

Figure 27 shows the structural features of the Xenopus NPR-C. XNPR-C presented a short intracellular tail, an unique transmembrane domain, and a extracellular portion characterized by the presence of 6 cystein residues identified in 2 intramolecular loops, and 2 intermolecular bounds, as described in Example VI, infra. In addition, the 3 potential sites for N-glycosylation are indicated by gray squares.

Figure 28 depicts the receptor binding studies on Xenopus oocytes injected with capped mRNA encoding XNPR-C receptor, as described in Example VI, infra.

Figure 29 illustrates the receptor binding studies on Xenopus oocytes injected with capped mRNA encoding XNPR-C receptor, as described in Example VI, infra.

Figure 30 depicts the modulation of cAMP contents by natriuretic peptides (A) or

neuropeptides (B) in oocytes overexpressing XNPR-C, as described in Example VI, infra.

Figure 31 illustrates the modulation of cAMP contents by neuropeptides (A) or natriuretic peptides (B) in oocytes overexpressing PAC-1, as described in Example VI, infra.

Figure 32 shows the modulation of cAMP contents by neuropeptides or natriuretic peptides in oocytes overexpressing XNPR-C and PAC-1, as described in Example VI, infra.

DETAILED DESCRIPTION OF THE INVENTION

The methods of the invention are based on the discovery that peptide receptor subtypes on neurons and related cells, including a new receptor, natriuretic peptide neuropeptide receptor or "NNPR," respond to compounds such as natriuretic peptides, for example atrial natriuretic peptide (ANP), brain natriuretic peptide (BNP), or type-C natriuretic peptide (CNP), as well as PHI-related neuropeptides, causing effects on the proliferation and/or survival of neurons. Proliferation, as used herein, means an effective increase in the number of cells treated with the compound of the invention e.g. natriuretic peptide as compared to non-treated cells. An effective increase in the number of cells so treated can occur as a result of an increase in survival, by an increase in mitotic rate, or a combination of both an increase in survival and an increase in mitotic rate. Prolonged or enhanced survival means that the cells exhibit longer individual survival rates and healthy function.

The invention is also based on the discovery of a novel receptor, NNPR, expressed on neuronal tumor cells, that interacts with natriuretic peptides, such as ANPs, as well as PHI-related neuropeptides such as PHI, VIP and PACAP.

The methods of the invention are also based on the finding that natriuretic receptor subtypes are expressed on developing Schwann cells and newly formed blood vessels in the developing brain, indicating that natriuretic peptides may also act indirectly on glial cells to regulate the proliferation and survival of neurons, or on blood vessel cells as they

invade new brain tissue or brain tumors.

The invention further provides a novel natriuretic peptide type-C receptor that is expressed in Xenopus oocytes and have shown ability to alter the signaling activity of unrelated receptors.

The effects of the compounds, such as natriuretic peptides, on proliferation of neurons and related cells, is a function of the subtypes and amounts of peptide receptors on the neurons, as well as the type and amount of endogenous natriuretic peptides present in the neuronal environment.

Cell types that may be regulated by the methods of the invention include but are not limited to normal and injured cells of the nervous system, such as neurons, Schwann cells, glial cells, oligodendrocytes, microglia, astrocytes, developing blood vessel cells, ganglion satellite cells, and immune cells involved in nerve injury or degeneration.

The invention provides methods and compositions for regulating the proliferation and/or prolonging the survival of neurons using compounds, such as natriuretic peptides, including ANPs, BNPs or CNPs, and/or PHI-related neuropeptides, such as PHI, VIP and PACAP, their analogues, derivatives or equivalents of these compounds, having the ability to stimulate or inhibit proliferation of neurons, and/or to prolong the survival of neurons, via interaction with their corresponding peptide receptors. The NNPR receptor may be used for a number of purposes, including, but not limited to use as a diagnostic and/or prognostic marker of neuronal cancers, generation of antibodies, and as targets for various therapeutic modalities, by directing agents to cancer cells via the NNPR.

The invention further provides methods and compositions for diagnosing the presence of neuronal tumors by detecting peptide receptors, such as type C receptors and the NNPR receptor of the invention, using labeled natriuretic peptides, PHI-related neuropeptides, or analogues, derivatives or fragments of these compounds.

The invention also provides methods for treating diseases or dysfunctions caused by injured or dying or dead neurons by increasing the proliferation and/or survival of the neurons, and for treating neuronal tumors by interfering with the proliferation and or survival of neuronal tumor cells using the compositions of the invention that stimulate peptide receptors such as NNPR on the cells, or inhibit type A or B receptors.

Still other methods of the invention are for prolonging survival of neurons used for transplants or for propagation of neurons *in vitro*.

The compounds of the invention also include agonists or antagonists of the natriuretic peptides and/or PHI-related neuropeptides, for example to interfere with the binding of endogenous natriuretic peptides and/or PHI-related neuropeptides, to their respective receptors on neurons. Thus, to inhibit neuronal tumor cell proliferation agonists of the peptides can be used to stimulate the NNPR receptor on the tumor cells. Alternatively, antagonists of the peptides, such as antibodies against natriuretic peptides or neuropeptides, or endopeptidases that digest such peptides, and including anti-sense oligonucleotides directed against the peptides or their receptors, can be used to reduce the availability of endogenous natriuretic peptides or interfere with the interaction of the peptides with type A or B receptors, which may be stimulating neuronal tumor growth. The compounds further include antagonists of the type A, B, C or C-like (e.g. NNPR) receptors, such as antibodies, that are administered to prevent the stimulatory or inhibitory responses of these receptors.

The guiding principle for carrying out the methods of the invention is to select agonists or antagonists of the natriuretic peptides and/or neuropeptides, and agonists or antagonists of their respective peptide receptor subtypes, to decrease proliferation of tumor cells, or to enhance proliferation and/or promote survival of healthy, damaged or dying neurons in vitro or in vivo, using the compositions of the present invention. Thus, where proliferation and enhanced survival of neurons is desired, for example, to ameliorate neurodegenerative disease, agonists of the natriuretic peptides such as ANPs, or of PHI-related neuropeptides

(e.g. PHI, VIP or PACAP), or the peptides themselves, are administered to stimulate proliferation via type A or type B receptors expressed on neurons. Production of endogenous peptides in vivo can also be addressed, using for example, agents that stimulate or inhibit expression of the peptides, such as anti-sense oligonucleotides directed against the peptides.

Where decreased proliferation is desired, for example to combat neuronal tumor growth, the response of peptide receptors such as subtype C or the NNPR receptor of the present invention, expressed on neuronal tumor cells, is stimulated using ligands for the receptors, such as natriuretic peptides and/or PHI-related neuropeptides, or their agonists.

Antibodies against natriuretic peptides are known (Clerico et al., J. *Endocrinol. Investi.*, 21:3 170-9 (1998)), Numata et al., *Clin. Chem.* 44:5, 1008-13 (1998)), as are antibodies against VIP related neuropeptides (Lundeberg and Nordlind, *Arch Dermatol Res.* 291(4):201-206 (1999)).

Moreover, methods for making antibodies, such as monoclonal antibodies, are well known in the art, using for example, hybridoma fusion techniques or by techniques that use EBV-immortalization methods. (See, e.g. Kohler and Milstein, *Nature,* 256:495-97 (1975); Brown et al., *J. Immunol.*, 127 (2):539-46 (1981); Brown et al., *J. Biol. Chem.*, 255:4980-83 (1980); Yeh et al., *Proc. Nat'l. Acad. Sci. (USA)*, 76 (6):2927-31 (1976); and Yeh et al., *Int. J. Cancer*, 29:269-75 (1982)).

Chimeric (mouse-human e.g., humanized antibodies) or human monoclonal antibodies may be preferable to murine antibodies for some therapeutic uses, because patients treated with mouse antibodies generate human anti-mouse antibodies. (Shawler et al., *J. Immunol.* 135:1530-35 (1985)). Chimeric mouse-human monoclonal antibodies reactive with the antigen can be produced, for example, by techniques recently developed for the production of chimeric antibodies (Oi et al., *Biotechnologies* 4(3):214-221 (1986); Liu et al., *Proc. Nat'l. Acad. Sci. (USA)* 84:3439-43 (1987)).

Novel antibodies of mouse or human origin can be also made to the antigen having the appropriate biological functions. For example, human monoclonal antibodies may be made by using the antigen, e.g. a natriuretic peptide, to sensitize human lymphocytes to the antigen in vitro followed by EBV-transformation or hybridization of the antigen-sensitized lymphocytes with mouse or human lymphocytes, as described by Borrebaeck et al. (*Proc. Nat'l. Acad. Sci. (USA)* 85:3995-99 (1988)).

Endopeptidases that digest natriuretic peptides are known (Delporte et al., *Eur. J. Pharmacol.* 207:81-88 (1991)).

Antagonists of the natriuretic peptides and PHI-related neuropeptides for use in the present invention include antisense oligonucleotides that block the expression of the genes encoding the peptides within cells by binding a complementary messenger RNA (mRNA) and preventing its translation (Wagner, Nature 372:332-335 (1994); and Crooke and Lebleu, *Antisense Research and Applications*, CRC Press, Boca Raton (1993)). Gene inhibition may be measured by determining the degradation of the target RNA.

Antisense DNA and RNA can be prepared by methods known in the art for synthesis of RNA including chemical synthesis such as solid phase phosphoramidite chemical synthesis or in vitro and in vivo transcription of DNA sequences encoding the antisense RNA molecule. The DNA sequences may be incorporated into vectors with RNA polymerase promoters such as the T7 or SP6 polymerase promoters. Alternatively, antisense cDNA constructs that synthesize antisense RNA constitutively or inducibly can be introduced into cell lines.

The potency of antisense oligonucleotides for inhibiting the target peptides may be enhanced using various methods including 1) addition of polylysine (Leonetti et al., *Bioconj. Biochem.* 1:149-153 (1990)); 2) encapsulation into antibody targeted liposomes (Leonetti et al., *Proc. Natl. Acad. Sci. USA* 87:2448-2451 (1990) and Zelphati et al.,

Antisense Research and Development 3:323-338 (1993)); 3) nanoparticles (Rajaonarivony et al., *J. Pharmaceutical Sciences* 82:912-917 (1993) and Haensler and Szoka, *Bioconj. Chem.* 4:372-379 (1993)), 4) the use of cationic acid liposomes (Felgner et al., *Proc. Natl. Acad. Sci. USA* 84:7413-7417 (1987); Capaccioli et al., *Biochem. Biophys. Res. Commun.* 197:818-825 (1993); Boutorine and Kostina, *Biochimie* 75:35-41 (1993); Zhu et al., *Science* 261:209-211 (1992) and Wagner, *Science* 280:1510-1513 (1993)); and 5) Sendai virus derived liposomes (Compagnon et al., *Exper. Cell Res.* 200:333-338 (1992) and Morishita et al., *Proc. Natl. Acad. Sci. USA* 90:8474-8478 (1993)), to deliver the oligonucleotides into cells. Recent techniques for enhancing delivery include the conjugation of the antisense oligonucleotides to a fusogenic peptide, e.g. derived from an influenza hemagglutinin envelope protein (Bongartz et al., *Nucleic Acids Res.* 22(22):4681-4688 (1994)).

Additional suitable antagonists of the peptides can be readily determined using methods known to the art to screen candidate agent molecules for binding to the peptides, such as assays for detecting the ability of a candidate agent to block binding of radiolabeled peptide to natriuretic receptors expressed on cells.

Additional embodiments of the invention include compounds that promote or interfere with the specific signaling pathways involved in the response of peptide receptors to the binding of natriuretic peptides, resulting in enhanced or decreased neuron proliferation and/or survival. Such compounds include those, which inhibit or promote the signaling pathway, for example by inhibiting a key compound in the pathway. For example, based on the data that PHI or type-C-like receptor-mediated inhibition of neuroblastoma proliferation occurs via inhibition of a MAP kinase pathway, MAP kinase inhibitors may be used to inhibit proliferation of neuronal tumors expressing these receptors. Alternatively, compounds such as cyclic GMP analogues can be used to increase proliferation and/or survival of neurons expressing type A or B (GC) peptide receptors.

Yet another embodiment of the invention includes a novel receptor, NNPR, that responds to

natriuretic peptides (e.g., ANP) and neuropeptides (e.g., PHI) that is expressed on neuronal tumor cells. The same, or another novel receptor is proposed based on the *in situ* hybridization results, *infra*, showing that gene expression for none of the three known receptors is detectable in a part of the develping brain that avidly binds radiolabeled natriuretic peptides. This receptor is isolated and cloned using methods known in the art. For example, the NNPR is cloned using either injection of in vitro transcribed mRNA into Xenopus oocytes, or by transfection of an eukaryotic expression library into mammalian cells. The Xenopus system is first evaluated for a response to PHI or desANP$_{4-23}$ after injection of polyA-selected RNA from Neuro 2a cells. The nature of specific current (calcium, chloride or potassium) is then determined to optimize the signal. RNA is then synthesized from the Neuro2a blastoma cell library constructed using Ziplock (BRL) phage vector (base = 3.0 x 10^6 independent clones, average insert size = 1.8 kb). This library contains efficient T7 and T3 polymerase sites, which allow for in vitro synthesis of mRNA. After injection of mRNA from this library elicits the response to PHI, the NNPR receptor cDNA is isolated by sib selection ((Simonsen and Lodish, *Trends Pharmacol. Sci.* 12:437-441 (1994)). If RNA from this library does not produce a desired response, the size of the NNPR receptor mRNA is determined and a new size-selected library is constructed (Simonsen and Lodish, supra). Alternatively, RNA may be used from another source, such as another neuroblastoma cell line or retinoic acid- or phorbol ester-differentiated Neuro 2a cells.

The NNPR receptor can be also cloned using expression cloning in mammalian cells such as COS cells using radioligand binding which depends only on high affinity binding of the radiolabeled ligand. Because the NNPR binds both PHI and desANP$_{4-23}$ with high affinity, either ligand or both can be used to detect expression. For example, the NNPR binds ^{125}I-PHI and ^{125}I-ANP, but is selectively displaced by either PHI or desANP$_{4-23}$. The method for cloning the NNPR uses methodology known in the art, such as that described by Ishihara et al., *EMBO J.* 10:1635-1641 (1991), used to isolate the secretin receptor cDNA. An existing neuroblastoma eukaryotic expression library can use cells, such as NG108 cells to clone the gamma opiate receptor (Evans et al., *Science* 258:1952-1955 (1992)), or SK-N-SH to clone

the plasma membrane noradrenaline transporter (Pachoiczyk et al., *Nature* 350:350-354 (1991)), or a library can be constructed from Neuro2a cells using an eukaryotic expression vector such as pcDNA (InVitrogen).

The library is screened using standard methods (Evans et al., supra). Briefly, 10 to 12 subdivisions are electroporated into COS cells. After transfection, cells are then transferred to tissue culture plates, and after three days, screened for PHI binding with ^{125}I-PHI. Plates are then washed and exposed to Cronex film (DuPont). After alignment of developed film with the plates, DNA from positive cells is extracted by the method of Hirt (*J. Mol. Biol.* 26:365-369 (1967)), transfected into competent bacteria, then retransfected into COS cells (Farnsworth et al., *Nature* 376:524-527 (1995)). This procedure can be repeated until a single clone is obtained. Alternatively, secondary and subsequent purification can be carried out using sib selection. The clone can be verified as encoding NNPR, if it recapitulates the correct radioligand binding/displacement profiles: e.g. it should bind ^{125}I-PHI, and be selective displaced by either PHI or desANP$_{4-23}$, and should also bind ^{125}I-ANP and be efficiently displaced by either PHI or desANP$_{4-23}$. Cells other than COS cells can be tested for expression of NNPR and used for cloning (Simonsen and Lodish, supra).

USES OF THE INVENTION

The compounds of the invention, such as natriuretic peptides, and/or PHI-related neuropeptides, their analogs, agonists, antagonists or derivatives thereof, may be used in pharmaceutical compositions for treating neuronal injury or diseases which result in neuronal degeneration, for example from stroke, brain, retina or spinal cord injuries, ischemia and reperfusion or other brain disorders and diseases as well as trauma associated with neurosurgical procedures or accidents. Brain disorders and diseases to be treated using the methods of the invention include, but are not limited to Huntington's disease, Alzheimer's disease, epilepsy, lathyrism, amyotrophic laterial sclerosis, and Parkinsonian dementia.

The compounds of the invention may also be used to promote survival and function of transplanted neurons.

The pharmaceutical compositions of the inventions can be administered as a homogenous solution, i.e. containing only one kind of peptide which recognize a receptor. Alternatively, the pharmaceutical composition can contain a heterogeneous solution, i.e. containing a combination of different natriuretic peptides or multiple natriuretic peptides and/or PHI related peptides, that recognize multiple different receptors.

The compounds of the invention may also be used to treat neuronal tumors and other forms of excessive neuronal cell proliferation, by decreasing proliferation and/or survival of the neurons. The novel NNPR receptor of the invention can also be used in methods of the invention. For example, this receptor can be used as a target for natriuretic peptides or neuropeptides, or mimics of these compounds, to inhibit proliferation of tumor cells expressing this novel receptor, and/or for ligands directed to the receptor for tumor cell killing, such as antibodies against the receptor, or radiotherapy using radiolabeled peptides.

Evaluation of the in vivo efficacy of the compositions of the invention to treat neuronal tumors can be achieved using animal tumor models, such as those known in the art (e.g. Cortes et al., *Gene Therapy* 5(11):1499-1507 (1998)). For example, neuroblastomas can be inoculated into an animal such as a rat. Subsequently, an anti-tumor composition of the invention, such as a natriuretic peptide, for example ANP, can be administered into the animal subject to stimulate type C receptors, and/or to stimulate the novel NNPR receptor of the invention to inhibit tumor cell proliferation.

Adminstration can be through any art recognized means. In one example, administration is by infusion, e.g., using a cannula connected by catheter tube to a mini-osmotic pump. The effects of the composition on tumor volume can be monitored, for example using magnetic resonance imaging (MRI) over periods of time to detect the size of the neuronal tumor in the animal.

Control animals can be used which also have neuronal tumors, but do not have the compositions of the invention administered. A decrease in the tumor volume over time relative to the tumor volume in untreated animals can indicate tumor regression as a result of administration of the compositions of the invention. These methods also lend themselves to gene therapy using retroviral vectors containing genes encoding the anti-tumor compounds of the invention, such as natriuretic peptides or PHI-related neuropeptides.

The methods of treating injured neurons or neuronal degeneration from diseases or injury, of enhancing neuron survival for transplants, or for treating neuronal tumors, include administering to a subject an effective dose of the compounds of the invention for interacting with peptide receptors, such as NNPR, on a cell. For example, neuronal tumors expressing the NNPR receptor of the invention and/or C-type receptors can be treated using radiolabeled peptides for radiotherapy, for example, or peptidase-resistant analogues of ANP and/or PHI-related neuropeptides.

For enhancing proliferation and/or survival of neurons, the effective dose will enhance the proliferation of neurons and/or prolong the survival of neurons, slowing neuronal degeneration or death, as compared to the proliferation and/or survival of neurons in an untreated subject. For treating tumors, the effective dose can result in a decrease in proliferation and/or survival of the neurons afflicted by the tumor, as compared to proliferation and/or survival of untreated neuronal tumor cells.

The compositions can be used in both the peripheral nervous system and the central nervous system. The compositions of the present invention can also be administered in combination with other compounds and agents for treating neuronal injury or disease, such as glutamate receptor antagonists or glucocorticoids.

For treating neurons in vivo in a subject, the compounds can be administered in a suitable pharmaceutical carrier in an effective dose so as to result in enhanced neuronal proliferation and/or survival. Alternatively, the compositions of the present invention can be administered in an amount effective to inhibit proliferation of neuronal tumors.

The peptides may also be introduced as nucleic acid encoding the gene for the compounds for expression in vivo. The nucleic acid can be directly introduced into a subject as "naked DNA", or is introduced in a retroviral vector, or cells containing recombinant vectors that include the nucleic acid, for use in gene therapy methods in vivo to increase proliferation and/or survival of neurons, for example to treat neurodegenerative diseases, or to prolong the survival of transplanted neurons.

The effective dosages and modes of administration are made in accordance with accepted medical practices taking into account the clinical condition of the individual subject (e.g. severity and course of the disease), the site and method of administration, scheduling of administration, patient age, sex, body weight and other factors known to medical practitioners. Accordingly, the dosages of the compositions of the invention for treatment of a subject are to be titrated to the individual subject. For example, the interrelationship of dosages for animals of various sizes and species and humans based on mg/m^2 of surface area is described by Freireich et al., *Cancer Chemother. Rep.* 50(4):219-244 (1966). The "effective dose" can be determined by procedures known in the art, and must be such as to achieve a discernible change in the proliferation and/or survival of neurons, including but not limited to increased neuronal proliferation over that observed in untreated neurons for enhancing survival of the neurons, or decreased proliferation over that observed for untreated neurons for treating neuronal tumors.

Methods are known in the art for determining efficacy of treatment of tumors, for example by measuring tumor regression over treatment time. Adjustments in the dosage regimen for treatment may be made to optimize the proliferation or inhibition, e.g. doses may be divided and administered on a daily basis, or the dose reduced proportionally depending on the

situation, e.g. several divided doses may be administered daily or proportionally reduced. The doses of the compositions of the invention required for treatments may be further refined with schedule optimization.

In one embodiment, the compositions of the invention are administered initially by intravenous injection to bring blood levels to a suitable level. The patient's levels of the compounds that interact with the natriuretic peptide receptor subtypes are then maintained by an oral dosage form, although other forms of administration, dependent upon the patient's condition and as indicated above, can be used. The quantity of compounds to be administered will vary for the patient being treated and will vary from about 100 ng/kg of body weight to 100 mg/kg of body weight per day and preferably will be from 10 mg/kg to 10 mg/kg per day.

The compositions can be administered in a variety of manners, in pharmaceutically acceptable forms, alone or as an active ingredient in combination with pharmaceutically acceptable carriers, diluents, adjuvants and vehicles. The compositions can be administered orally, subcutaneously or parenterally, including intravenous, intraarterial, intramuscular, intraperitoneally and intranasally, as well as using intrathecal and infusion techniques depending on dosing requirements and other factors known to those skilled in the art. Implants of the compounds may also be useful.

When administering parenterally, the compositions of the invention will generally be formulated in a unit dosage injectable form (solution, suspension, emulsion). The pharmaceutical formulations suitable for injection include sterile aqueous solutions or dispersions and sterile powders for reconstitution into sterile injectable solutions or dispersions. The carrier can be a solvent or dispersing medium containing, for example, water, ethanol, polyol (for example, glycerol, propylene glycol, liquid polyethylene glycol, and the like), suitable mixtures thereof, and vegetable oils.

Proper fluidity can be maintained, for example, by the use of a coating such as lecithin, by the maintenance of the required particle size in the case of dispersion and by the use of surfactants. Nonaqueous vehicles such a cottonseed oil, sesame oil, olive oil, soybean oil, corn oil, sunflower oil, or peanut oil and esters, such as isopropyl myristate, may also be used as solvent systems for compound compositions. Additionally, various additives which enhance the stability, sterility, and isotonicity of the compositions, including antimicrobial preservatives, antioxidants, chelating agents, and buffers, can be added. Prevention of the action of microorganisms can be ensured by various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, sorbic acid, and the like. In many cases, it will be desirable to include isotonic agents, for example, sugars, sodium chloride, and the like. Prolonged absorption of the injectable pharmaceutical form can be brought about by the use of agents delaying absorption, for example, aluminum monostearate and gelatin. According to the present invention, however, any vehicle, diluent, or additive used would have to be compatible with the compounds.

Sterile injectable solutions can be prepared by incorporating the compounds utilized in practicing the present invention in the required amount of the appropriate solvent with several of the other ingredients, as desired.

A pharmacological formulation can be administered to the patient in an injectable formulation containing any compatible carrier, such as various vehicle, adjuvants, additives, and diluents; or the compounds utilized in the present invention can be administered parenterally to the patient in the form of slow-release subcutaneous implants or targeted delivery systems such as monoclonal antibodies, vectored delivery, iontophoretic, polymer matrices, liposomes, and microspheres. Examples of delivery systems useful in the present invention include: U.S. Pat. No. 5,225,182; U.S. Pat. No. 5,169,383; U.S. Pat. No. 5,167,616; U.S. Pat. No. 4,959,217; U.S. Pat. No. 4,487,603; U.S. Pat. No. 4,486,194; U.S. Pat. No. 4,447,233; U.S. Pat. No. 4,447,224; U.S. Pat. No. 4,439,196; and U.S. Pat. No. 4,475,196. Many other such implants, delivery systems, and modules are well known to those skilled in the art.

A pharmacological formulation of the compounds utilized in the present invention can be administered orally to the patient. Conventional methods such as administering the compounds in tablets, suspensions, solutions, emulsions, capsules, powders, syrups and the like are usable.

Known techniques which deliver the compositions of the invention orally, subcutaneously or parenterally including intravenous, intraarterial, intramuscular, intraperitoneally, and intranasal administration as well as intrathecal and infusion techniques and retain the biological activity are preferred.

For delivery within the CNS, intrathecal delivery can be used with, for example, an Ommaya reservoir. U.S. Pat. No. 5,455,044 provides for use of a dispersion system for CNS delivery or see U.S. Pat. No. 5,558,852 for a discussion of CNS delivery. Pharmacological formulations that cross the blood-brain barrier can be prepared using known methods. Such formulations can take advantage of methods now available to produce chimeric peptides in which the compounds of the present invention are coupled to a brain transport vector allowing transportation across the barrier. (Pardridge, et al., *West J. Med.*, 156(3):281-286 (1992); Pardridge, *Pharm. Toxicol.* 71(1):3-10 (1992); Bickel, et al., *PNAS (USA)* 90(7):2618-2622 (1993), or methods of gene therapy (Kramer et al., *Nature Med.* 1:1162-1166 (1995). Alternatively, direct infusion into the cerebral spinal fluid can also be undertaken (Wilkins and Rengachary, *Neurosurgery*, Vol. II, Ch. 190 (McGraw Hill), pp 1544-1570 (1982). Additionally, blood-brain-barrier disruption may be used in appropriate cases (Neuwelt et al., *Ann. Int. Med.* 93:137-139 (1980).

It is to be noted, that in the case of head injury (brain injury) that the blood brain barrier is generally "breached" in the area of the trauma and the compositions of the present invention can also be administered at the site of injury thereby delivering them directly to the site.

Alternatively, gene therapy utilizing the gene for compounds that interact with natriuretic peptide receptor subtypes can be employed using known gene therapy protocols. The present invention provides a method of gene therapy for treating diseases which result in neuronal degeneration resulting from damage and death by administering the gene for the compounds so that the compounds are produced *in situ* where needed to counteract the damage or disease process. For example, pre-engineered T lymphocytes that contain the cloned gene under a strong promoter and that overexpress therefore, the gene product, can be used to deliver the compounds that interact with the natriuretic receptor subtypes on neurons to neural tissue (Kramer et al., supra, 1995).

The present invention provides for gene therapy utilizing vectors comprising an expression control sequence operatively linked to the nucleic acid sequence of genes for the compounds of the invention that interact with natriuretic peptide receptor subtypes on neurons for therapeutic uses.

Vectors are known or can be constructed by those skilled in the art and should contain all expression elements necessary to achieve the desired transcription of the sequences. In particular, see the methods set forth in U.S. Pat. No. 4,866,042 to Neuwelt issued Sep. 12, 1989 titled "Method for the delivery of genetic material across the blood brain barrier" incorporated herein in its entirety by reference.

The vectors can be introduced into cells or tissues by any one of a variety of known methods within the art. Such methods can be found generally described in Sambrook et al., Molecular Cloning: A Laboratory Manual, Cold Springs Harbor Laboratory, New York (1989, 1992), in Ausubel et al., Current Protocols in Molecular Biology, John Wiley and Sons, Baltimore, Md. (1989), Chang et al., Somatic Gene Therapy, CRC Press, Ann Arbor, Mich. (1995), Vega et al., Gene Targeting, CRC Press, Ann Arbor, Mich. (1995), Vectors: A Survey of Molecular Cloning Vectors and Their Uses, Butterworths, Boston, Mass. (1988) and Gilboa et al, *BioTechniques* 4(6):504-512 (1986) and include, for example, stable or transient transfection, lipofection, electroporation and infection with recombinant

viral vectors. In addition, see U.S. Pat. No. 4,866,042 for vectors involving the central nervous system and also U.S. Pat. Nos. 5,464,764 and 5,487,992 for positive-negative selection methods.

Recombinant viral vectors are an example of vectors useful for in vivo expression of a desired nucleic acid because they offer advantages such as lateral infection and targeting specificity. The vector to be used in the methods of the invention will depend on desired cell type to be targeted. For example, if neural cells are to be treated, then a vector specific for such neural cells will be used. Alternatively, cells can be transformed that target to the central nervous system.

Retroviral vectors can be used. The vector's genome can be also engineered to encode and express the desired recombinant gene. The specific type of vector will depend upon the intended application. Retroviral vectors are also known and readily available within the art or can be constructed by one skilled in the art using well-known methodology.

If viral vectors are used, for example, the procedure can take advantage of their target specificity and consequently, do not have to be administered locally at the diseased site. However, local administration may provide a quicker and more effective treatment, administration can also be performed by, for example, intravenous or subcutaneous injection into the subject. Injection of the viral vectors into a spinal fluid can also be used as a mode of administration, especially in the case of neurodegenerative diseases. Following injection, the viral vectors will circulate until they recognize host cells with the appropriate target specificity for infection.

Transfection vehicles such as liposomes can also be used to introduce the non-viral vectors described above into recipient cells within the inoculated area. Such transfection vehicles are known by one skilled within the art.

The methods and compositions of the present invention are useful to regulate the proliferation and/or to prolong the survival of neurons, and in particular to stimulate proliferation and/or promote survival of neurons that are injured or degenerating, for example from diseases such as Alzheimer's, or to prolong survival of transplanted neurons. The methods and compositions are also useful to inhibit proliferation of neuronal tumors.

In neurons that express stimulatory peptide receptors, such as type A and/or type B receptors, administration of compounds such as ANPs, will result in stimulation of proliferation or enhanced survival of the neurons, which can be used to treat, for example, nerve injury or neurodegenerative disease. In the mammalian adult brain, type B receptors predominate, such that administration of compounds such as ANPs that interact so as to stimulate B receptors should result in stimulation of proliferation or enhanced survival of the neurons present in that region.

In neurons that express type C receptors, such as tumors, administration of the ANP compositions of the invention will result in inhibition of proliferation and/or survival of neurons via response of the type C receptors. Such inhibition can lead to tumor regression.

In neurons that express multiple receptors that are inhibitory and stimulatory, for example cells that express A, B, and C and C-like (e.g. NNPR) peptide receptors, such as the neuroblastoma cell experiments described in the Example, infra, administration of ANPs at high doses, e.g. in the range of 10 nM to 1 μM will result in inhibition of proliferation of the tumor cells as a result of response of the C and C-like receptors. Administration of low doses of ANPs, e.g. in the subnanomolar range (1 picomole to 1 nM) in such cells, will result in stimulation of proliferation or enhanced survival of such cells via the response of A and B receptors.

The methods of the invention include the administration of compounds to block endogenous natriuretic peptides in vivo in order to prevent binding to type A or B receptors on cells where such binding results in increased proliferation of tumor cells. Such agents

include antibodies against natriuretic peptides, for example anti-ANP antibodies and anti-PHI-related neuropeptides.

There are multiple diagnostic uses of the invention. For example, the invention provides methods for diagnosing in a subject, e.g., an animal or human subject, a cancer associated with the presence an NPR receptor. In one embodiment, the method comprises quantitatively determining the NPR receptor in the sample (e.g., cell or biological fluid sample) using any one or combination of antibodies reactive with NPR. Then the amount of NPR receptor so determined can be compared with the amount in a sample from a normal subject. The presence of a measurably different amount (i.e., the amount of NPR receptor in the test sample exceeds the number from a normal sample) in the samples suggesting the presence of the neuronal cancer. The progress of the tumor can be monitored, as can efficacy of treatment using such methods.

Further, antibodies against NPR receptors can be used in diagnostic kits comprising an antibody that recognizes and binds NPR receptor; and a conjugate of a detectable label and a specific binding partner of the anti-NPR antibody. In accordance with the practice of the invention the label includes, but is not limited to, enzymes, radiolabels, chromophores and fluorophores.

For example, antibodies reactive with a NPR receptor of the invention may be particularly useful in diagnostic assays, imaging methodologies, and therapeutic methods in the management of neuronal cancer. Diagnostic assays generally comprise one or more monoclonal antibodies capable of recognizing and binding NPR, and include various immunological assay formats well known in the art, including but not limited to various types of precipitation, agglutination, complement fixation, radioimmunoassays (RIA), enzyme-linked immunosorbent assays (ELISA), enzyme-linked immunofluorescent assays (ELIFA) (H. Liu et al. *Cancer Research* 58: 4055-4060 (1998), immunohistochemical analysis and the like. In addition, immunological imaging methods capable of detecting neuronal cancer can be used, including but limited to radioscintigraphic imaging methods

using labeled NPR antibodies. Such assays may be clinically useful in the detection, monitoring, and prognosis of neuronal cancer. For example, NPR receptors may be detected in tumor biopsies to assess in staging and prognosis.

Antibodies against NPR can also be used therapeutically to target neuronal cancer cells when used alone or conjugated to a radioisotope or toxins.

The following examples are presented to illustrate the present invention and to assist one of ordinary skill in making and using the same. The examples are not intended in any way to otherwise limit the scope of the invention.

EXAMPLE I

This example demonstrates the proliferative action of natriuretic peptides on neuroblastoma Neuro2a cells.

Methods

Cell cultures. Neuro2a cells (ATCC, Rockville, MD) were maintained in 75 cm^2 flasks (Falcon, South Dakota) in 20 ml of Dulbecco's Modified Eagle Medium (DMEM) (Cellbio/Fisher). Medium was supplemented with 8% fetal bovine serum (FBS) (Gibco-BRL/Life Technology, Gaithersburg, MD) and antibiotics (penicillin/streptomycin) under a 5% CO_2/ 95% air controlled atmosphere at 37° C. The medium was changed every 3 days. Passages were performed after trypsinization (0.05% trypsin /0.53 mM EDTA, Gibco-BRL/life technology Gaithersburg, MD).

Proliferation studies. Cells were seeded in 24-well plates (80,000 cells/well) in 1 ml medium and cultured for 24 h. Medium was then replaced with fresh serum-free medium. After 1.5 h at 37° C, cells were preincubated for an additional hour with vehicle or signal transduction inhibitors or receptor antagonists (GF109203, H89 and Rp-8-pCTP-cGMPS

from Calbiochem, and PD98059 from New England Biolabs). The NPRA/NPRB antagonist HS-142-1 was kindly provided by Kyowa Hakko Kogyo Co., Shizuoka, Japan. Peptides ($desANP_{4-23}$ obtained from Sigma Chemical Co. (St. Louis, MO), ANP, CNP, and VIP from Sigma, St. Louis, MO) were added, and then 1h later ^{3}H-thymidine (1 :Ci/well) was added. Four hours later ^{3}H-thymidine incorporation was determined as previously described (Lelievre et al., *J. Biol. Chem.* 31:19685-19690 (1998)) using TCA precipitation.

Binding studies. ^{125}I-ANP (2200 Ci/mmol), ^{125}I -PACAP-27 were obtained from Dupont/NEN . Because ^{125}I -PHI was not commercially available, PHI was radio-iodinated with the chloroamine -T technique as described by Paul et al., *Life Sci.* 41:2373-3480 (1987)). Iodinated peptide was separated by reverse-phase HPLC (Spectraphysics) using a 5 : VYDAC C18 column (Interchrom, France). A gradient of acetonitrile, H_2O, 0.1% trifluoroacetic acid was required for elution. Fractions containing ^{125}I PHI were evaporated under nitrogen and stored at -20°C. Binding experiments for PACAP and PHI were carried out as described by Lelievre et al., *J. Biol. Chem.* 31:19685-19690 (1998)). Cells were seeded at 80,000/ml/well in 24-well dishes and cultured for three days. Fifteen min before the initiation of the binding, cells were incubated in fresh medium at 37°C. Culture medium was then removed and replaced with 270 µl of cold binding medium containing 30,000 cpm of radiotracer (^{125}I-VIP and ^{125}I-PACAP, 2200 Ci/mmol from NEN Life Science Products) and 30 µl of specified concentrations of unlabeled peptides (Lelievre et al., *Neuropeptides* 30:313-322 (1996)). Radioiodinated PHI binding experiments were performed on intact cells in suspension, using a slightly different procedure to increased sensitivity. Nonconfluent cultures were harvested by incubation for 5 min at 37°C in a phosphate buffer (0.05M, pH 7.4) containing 0.53 mM EDTA. Cells were then quickly centrifuged, pellets were reconstituted in the binding buffer containing 35,000 cpm of ^{125}I-PHI and specified concentrations of the unlabeled ligands, and then cells, were incubated under shaking. The incubation times were determined by time course experiments conducted at 4°C and then fixed at 120, 150 and 210 min for VIP, PACAP and PHI displacements, respectively. Incubation was stopped with a quick rinse with 2ml of cold phosphate-buffered saline

containing 0.1% bovine serum albumin, followed by cell lysis in 0.5 M NaOH solution. Extracts were transferred into 5-ml tubes and radioactivity was counted using a gamma counter (Wallac). For ^{125}I -ANP, displacements were conducted on suspended cells in Eppendorf tubes (800,000 cells/ml/well) in binding medium which contained 25,000 to 35,000 cpm of ^{125}I-ANP and the specified concentrations of competitive analogs. Incubation was performed at 4°C under gentle agitation for 150 minutes. Rinses and bound radioactivity measurements were determined as for the ^{125}I-PHI displacement experiments above.

Cyclic AMP and GMP measurements. Cells (80,000/well) were cultured for 3 days in 24-well plates. Medium was replaced with serum-free medium with or without 10 μM isobutylmethylxanthine (IBMX) or 2 μM forskolin (Sigma, St. Louis, MO). After incubation for 15 min at 37°C, peptides were added and incubated further for 15 min at 37°C. Cells were lysed in 6% TCA solution and radioimmunoassays were performed following recommendations of the manufacturer (Dupont-NEN Life Science Products).

Intracellular calcium fluorometry Cells were cultured for 3 days on glass coverslips until 80% confluency was reached. Then cells were incubated in culture medium for 2h at room temperature in the presence of 5 μM fura-2/AM. Cells were rinsed twice in HBSS medium containing 0.1% BSA, $MgSO_4$, $CaCl_2$, $NaHCO_3$ and $MgCl_2$ (Gibco-BRL/Life Technology, Gaithersburg, MD). Measurements were performed at 30°C for 300 s on a Hitachi F2000 fluorescence spectrophotometer. Twenty microliters of specified peptides and/or calibration reagents (15:1 of 5 mM digitonin and 50:1 of 0.2 mM EGTA/pH 8.5) were added into the stimulation buffer (2 ml) after 5 min equilibration. The intensity of fluorescence at 340 and 380 nm was converted into calcium concentration.

Purification of Total RNA and RT-PCR

Total RNA was extracted from neural tubes obtained from mouse E10 embryo as described by Waschek et al., *PNAS (USA)* 95:9602-9607 (1998), incorporated by

reference herein. Briefly, neuroepithelial cells were obtained as follows: on the specified day (e.g. 10 days after mating) pregnant mice were sacrificed, embryos were removed from the uterus and incubated in culture medium (CO_2 -independent medium from Gibco/BRL, Gaithersburg, MD). Embryos were extracted from placenta and yolk sac by teasing. "Naked" embryos were transferred into a new plate containing fresh medium. Using surgical scissors, neural tubes from the midbrain to the upper part of the spinal cord were excised. Neural tubes were then transferred into a dissociation solution (CO_2 independent medium supplemented with 1 mg/ml of trypsin and 10 μg/ml of DNAse) and incubated at 37°C for 12 min. Enzymic digestion was stopped by transferring the neural tube into a stop buffer (CO_2 independent medium containing 5% serum). In this buffer, neural tubes were dissected. First the tube (not closed at E10 at the level of the hindbrain) was incised to form a layer of tissue, then the two layers of tissues constituting the tube (neuroepithelial cell layer inside and mesoderm layer outside) were separated using fine tweezers. The mesoderm was discarded, while the neuroepithelial cell layer was transferred into a washing buffer (Hank's solution, Gibco, Gaithersburg, MD). After spinning down the layers, the washing buffer was replaced by a trypsin solution for a 10 min incubation at 37°C, and digestion was stopped by addition of 10% FCS (fetal calf serum). The cell suspension was spun down again and the trypsin solution was washed twice with 10 ml of culture medium. The cells were then plated out in 5 ml of culture medium (Neurobasal medium from Gibco, Gaithersburg, MD) containing Penicillin/streptomycin, 1X N2 supplement (Gibco, Gaithersburg, MD) and 10 ng/ml of bFGF (basic Fibroblast growth factor) (Sigma, St. Louis, MO). Cells were cultured overnight and dividing cells (pseudoganglions) were subcultured for PCR as described below.

RT-PCR: PolyA$^+$-selected mRNAs from Neuro2a cells or mouse tissues were reverse-transcribed into cDNA and amplified by polymerase chain reaction (PCR), using the GeneAmp kit supplied by Perkin Elmer. Primers were designed using the Primer3 software, from mouse or rat natriuretic-receptor sequences published in the NCBI database, and were synthesized by BRL-Gibco. The initial set of selected sense primers were 5'-

ATTTGTGGGAGCTTGTACCG-3', 5'-GTGTACCCTGCTGCCTCTGT-3 and 5'-CTTCCAGGTGGCCTACGAA-3' and antisense primers were 5'-GGCAATTTCC TGAAGGATGA-3', 5'-CCGCAGATATACACAATGCG-3' and 5'-GGCACACATGAT CACCACTC-3' for natriuretic receptor subtypes A, B, and C, respectively. These were designed to generate PCR fragments of 389, 379 and 492 bp, corresponding to nucleotides 1790-2179, 376-755 and 279-771 for natriuretic receptor subtypes A, B, and C respectively. Amplifications were carried out for 35 cycles of denaturation (94°C, 50 s), annealing (54°C, 45 s) and extension (72°C, 45 s). PCR was finished by a step of 5 min at 72°C. Single products of the expected size were amplified from Neuro2a cell RNA (corresponding to the receptor subtypes A and B), or from mouse kidney (receptor subtype C).

To validate the nature of the PCR products, one-fifth of the PCR products were run on 2% agarose gels, followed by overnight transfer to nylon membrane (Magna, MSI). Membranes were baked, UV cross-linked and then preincubated at 37°C, for 6 hours in the hybridization solution described by Waschek et al., Peptides 18:835-841 (1997), incorporated by reference herein ((50% formamide, 5X SSC, 5X Denhardt's solution, 0.05M $NaHPO_4$, 0.1% SDS, tRNA 0.2 g/l, HSDNA 0.1 g/l). The following ^{32}P-end-labelled internal oligonucleotides: 5'-GCGTGGTAGATGGACGTTTT-3', 5'-GTATCTGGATGCTCGCACAG-3' and 5'-CTGGA CGACATAGTGCGCTA-3'. These corresponded to the nucleotides 1996-2015, 579-598 and 715-734 of the sequences of natriuretic receptor subtypes A, B, and C, respectively (Genbank accession numbers L31932, X14177 and D78175, respectively). Membranes were washed three times at 42°C for 20 min in 1X SSC buffer containing 0.1 % SDS. Signals were detected using a Phosphorimager (Molecular Dynamics) (exposure time from 2h to overnight). In parallel, PCR products were subcloned into PCR2.1 vector using a TA cloning kit (Invitrogen, San Diego, CA), and sequenced to confirm their identity.

To further study the possible expression of NPRC in Neuro2a cells, numerous primers were designed covering almost the entire sequence of the mRNA (Figure 7A). PCR products obtained by amplification of Neuro2a samples were compared with that from embryonic

neural tube. Three primers named respectively, OMPRC1+ 5'-CTTCCAGGTGGCCTACGAA-3', OMPRC2+, 5'-CTGGACGACATAGTGCGCTA-3'; OMPRC3- 5'-GGCACACATGATCACCACTC-3' (located in positions 290-309, 714-734 and 771-751, respectively) were the same as previously used to amplify the NPRC from mouse kidney. Consensus primers were also designed I highly conserved regions between mouse, human, bovine and eel NPRC sequences. These oligonucleotides were named OPRCX1+ (5'TGAGGACAGCG AAACCTGAGTT-3'), OPRCX2+ (5'-ATGTTTGTTGAAGGATTCCA-3'), OPRCX1- (5'-GCAGATTCTTCTAGGCCAC-3', OPRCX2- (5'TTCTTCCTGAAAAGTAGAAGGC-3'), corresponding to the mouse sequences 959-980, 1051-1070 and 1505-1483, respectively. Amplifications were carried out for 40 cycles of denaturation (94°C, 40 s), annealing (50-52°C, 45 s) and extension (72°C, 45 s). PCR was finished by a step of 5 min. at 72°C. Two different concentrations of $MgCl_2$ (2 and 3 mM) were tested for each set of primers. To validate the nature of the PCR products, one-fifth of the PCR products were run on 2% agarose gels, followed by overnight transfer to nylon membrane (Magna, MSI). Membranes were baked, UV cross-linked and then preincubated at 37°C, for 6 hours in the hybridization solutions. Hybridizations were performed at 37°C for 6 hours, using the following ^{32}P-end-labelled internal oligonucleotides OMPRC2+ (for the set OMPRC1+/ OMPRC3- and OPRCX4+ for the others (5'-CGAATGTCAAATATCCTTGGGG-3' that corresponds to the mouse sequence 1322-1343). Three rinses were performed using a low stringency buffer (1XSSC/0.1% SDS) at 40°C. Signals were detected using a Phosphorimager (Molecular Dynamics) (exposure time from 2 h to overnight).

Purification of mRNA for Northern and Southern blots. Neuro2a cells were cultured in 8 flasks (75 cm^2) and harvested at 90 % confluency with 0.05 % trypsin/0.02 % EDTA. Trypsin was neutralized with the tissue culture medium (containing 8% serum), cells were centrifuged and then rinsed with phosphate-buffered saline (PBS). Subsequent isolation of polyA-selected mRNA was carried out as described by Waschek et al., *Peptides* 18(6):835-841 (1997). Poly A RNA was isolated using the Fast Track 2.0 kit (InVitrogen, San Diego, CA). RNA was quantified by UV absorbance and run on 1.2%

agarose gels.

Northern Analysis Total RNA from mouse brain and polyA-selected mRNA from mouse Neuro2a neuroblastoma cells, kidney and spleen tissues were loaded at the concentration of 15 μg/lane on 0.8% agarose 2% formaldehyde MOPS gel. After transfer to nylon membranes (Magna, MSI), blots were prehybridized overnight and then hybridized for 8 hours at 45°C with cloned natriuretic receptor cDNA probes originally obtained from PCR experiments. EcoR1-generated probes were labeled by random incorporation of ^{32}P-dCTP (Random prime kit from Gibco-BRL, Gaithersburg, MD). After 3 washes for 20 min at 54°C in 0.2X SSC buffer containing 0.1 % SDS, blots were exposed for 3 days and signals detected with a Phosphorimager (Molecular Dynamics).

Results

Action of ANP analogs on proliferation

Assay of thymidine incorporation over a 5 hr period of peptide treatment revealed a dose-dependent biphasic action of ANP and CNP on neuroblastoma cells (Figure 1A). At concentrations below 10 nM, ANP and CNP slightly, but significantly, stimulated proliferation (maximal increase observed, 13%), whereas they more strongly inhibited cell proliferation at higher doses. At concentrations of 1μM of ANP and CNP, inhibition reached about 34 and 28% of control, respectively. The NPRC selective analog desANP$_{4-23}$ did not stimulate proliferation at any concentration, and produced the strongest degree of inhibition (43%) with an IC_{50} of about 23 nM (Figure 1A). Effects of natriuretic peptides were unaffected by O-phenanthrolin and bacitracin, which have been shown to potently inhibit endopeptidase activities that cleave natriuretic peptides in other neuroblastoma cells (Delporte C. et al., *Eur J Pharmacol*, 227:247-56 (1992)), indicating that the actions of ANP analogs were not due to ANP degradation products.

To obtain information regarding the signaling pathways used by natriuretic peptides to control cell proliferation, peptide actions were studied in the presence and absence of various protein kinase (PK) inhibitors. These included inhibitors of PKA: H89 (20 μM) (Chijiwa T. et al., *J Biol Chem*, 265, 5267-5272 (1990)), PKC: GF109203X (10 μM) (Toullec D. et al., *J Biol Chem*, 266, 15771-15781 (1991)), MEK1/2 kinase: PD98059 (20 μM) (Alessi DR et al., *J Biol Chem*, 270: 27489-27494 (1995)) and PKG: Rp-8-pCTP-cGMPS (20 μM) (Zhuo M. et al., *Nature*, 368: 635-639 (1994)). PKA and PKC inhibitors did not significantly alter the inhibitory actions of any of the natriuretic analogs tested (Figures 1B and 1C, respectively). In contrast, PD98059 completely inhibited the anti-proliferative actions of ANP, CNP and desANP$_{4-23}$ (Figure 1D), resulting in stimulation rather than inhibition of proliferation at ANP and CNP concentrations of 10 nM and above. Conversely, PKG inhibitor completely blocked the ANP and CNP-induced stimulatory effects, and enhanced the inhibitory effects of ANP peptides (Figure 1E).

The potent action of NPRC-specific agonist desANP$_{4-23}$ suggested that these cells express a type C receptor coupled to growth inhibition. Although lower concentrations of ANP and CNP slightly stimulated proliferation via GC receptors, higher concentrations (10 nM and greater) inhibited growth, perhaps due to over-riding action on a type C (non GC) receptor. The possibility that a type C receptor mediated the growth-inhibitory actions was supported by the fact that antiproliferative effect of each natriuretic peptide was insensitive to PKG blockade. Instead, all inhibitory actions were selectively sensitive to MAP kinase pathway blockade. Nonetheless, NPRA or NPRB could couple to an alternative signaling pathway in the presence of higher concentrations of natriuretic peptides. To test for this possibility, DNA synthesis was determined in cells incubated with antiproliferative concentrations of natriuretic peptides in the presence and absence of the NPRA/NPRB-selective antagonist HS-142-1 (Matsuda Y., *Humana Press Inc, Totowa NJ (Samson WK and Levin ER eds)*, chapt. 17, pp. 289-307 (1997)) (Figure 2). The antiproliferative actions of ANP, CNP, and desANP$_{4-23}$ were all unaffected by this drug, indicating that the growth-inhibitory effects were probably not mediated by NPRA or NPRB. In addition, HS-142-1 had a only a negligible effect on DNA synthesis in untreated cells indicating Neuro2a cells produce little,

if any, natriuretic peptides capable of interacting with NPRA or NPRB under these conditions.

Pharmacological characterization of natriuretic receptors

Displacement of ^{125}I-ANP from Neuro2a cells was performed using increasing concentrations of ANP, CNP and ANP_{4-23}, as well as PHI-related peptides PHI, VIP, and PACAP, which has been proposed to interact with natriuretic receptors (Murthy KS et al., *Am J Physiol*, 275:C1409-1416 (1998); Brown J et al., *Am J Physiol*, 269: R261-R273 (1995)). Data were fit to a sigmoidal equation (Fig.3A). IC_{50} and Hill slope values are given in Table I. All natriuretic peptides except PACAP-27 completely displaced ^{125}I-ANP with an IC_{50} value in the low nanomolar or subnanomolar range. The Hill values were generally less than 0.8, suggesting the presence of more than one ^{125}I-ANP binding site. The IC_{50} values gave the following pharmacological profile: $desANP_{4-23}$ <CNP<PACAP-38<VIP <PHI<ANP<<PACAP-27. Approximately 65% of ANP binding was competitively inhibited by the selective NPRA/NPRB inhibitor HS142-1, with an IC_{50} of about 7 μg/ml (Figure 4A). In the presence of concentrations of HS142-1 which maximally displaced ^{125}I-ANP, $desANP_{4-23}$ was still able to specifically displace ^{125}I-ANP binding (about 35% of total binding in the absence of HS142-1), with an IC_{50} of about 22"3.5 pM (Figure 4B). These binding data suggest that approximately 65% of ^{125}I-ANP binding sites are type A or B, whereas 35% are type C.

TABLE I

	^{125}I-ANP displacement		^{125}I-PACAP displacement		^{125}I-PHI displacement	
Ligands	IC_{50}±SEM (nM)	Hill slope (±SEM)	IC_{50} (pM) Site 1	IC50 (nM) Site 2	IC_{50} (pM)	IC50 (nM)
ANP	3.45±0.25	0.94±0.2	NA (>70%)	117 ± 2.6	592 ± 14	NA (80%)
CNP	0.28±0.18	0.78±0.18	NA (>70%)	73 ± 1.4	NA	NA
DesANP $_{4-23}$	0.131±0.18	0.76±0.15	NA (>70%)	318 ± 23	156 ± 3.6	NA (100%)
PHI	1.9±0.2	0.53±0.05	*62 ± 0.8	*NA (37%)	*203± 12	*NA (100%)
VIP	0.78±0.25	0.65±0.11	*25 ± 0.6	*NA (25%)	*51 ± 1.65	*NA (55%)
PACAP-27	327±2.4	0.70±0.25	*20 ± 0.3	*52 ± 2.3	*4.7 ± 2.1	*61 ± 6.5
PACAP-38	0.36±0.28	0.60±0.15	*3.7 ± 0.4	*5.7 ± 1.8	*40 ± 2.8	*60 ± 5.2

Table I shows the results of displacement of ANP, PACAP and PHI radioligands by unlabelled analogs in Neuro2a cells. All values (including mean ± SEM) were obtained from the data in Figure 3. N.A. = not available. * Data reported in Lelievre et al., *J. Biol. Chem.* 273:19685-19690 (1998)). If ligands displaced only partially the radiotracer binding, % is indicated in brackets.

Common binding sites for ANP, PACAP AND PHI

The above results show that PHI and VIP analogs can displace the binding of the ANP radioligand, supporting the idea of common ANP/PHI/VIP sites. This was reinforced by the data of Figure 3B and C, demonstrating that ANP analogs are also capable of displacing the binding of ^{125}I-PACAP as well as of ^{125}I-PHI. ^{125}I-PHI binding was totally displaced by both desANP$_{4-23}$ and PHI with a similar high affinity (IC50: 0.203 nM vs. 0.156 nM, for PHI and desANP$_{4-23}$, respectively), whereas ANP displaced only 80% of the total binding.

Coupling to cyclic nucleotide messengers and intracellular calcium

Natriuretic peptide-induced changes in cGMP levels were measured in Neuro2a cells in the presence of 10 μM IBMX to inhibit phosphodiesterase activity. ANP and CNP potently triggered up to a 6-fold increase of cGMP levels, whereas $desANP_{4-23}$ and PHI did not produce any measurable change (Figure 5). Moreover, PHI at 50 nM, a concentration that did not increase cGMP or cAMP, did not alter the ANP-induced cGMP elevation.

Type C receptors have been shown to couple to adenylate cyclase inhibition and calcium influx in other systems (Hirata M. et al., *Biochim Biophys Acta*, 1010: 346-351 (1989); Maack T. et al., *Science*, 223:675-678 (1987)). Thus peptide-induced changes in cAMP levels were measured in the presence of 2 μM forskolin. None of the ANP analogs effected cAMP levels under these conditions. As previously reported, PHI induced activation only at concentrations over 100 nM.

Changes in intracellular calcium levels were measured in Neuro2a cells grown on glass coverslips, in response to 0.1, 10 or 20 nM of natriuretic peptides ANP, CNP, and $desANP_{4-23}$. None of the natriuretic peptides induced changes in intracellular calcium levels (Figure 6). In contrast, other neuropeptides (1 nM PACAP-38, 10nM PACAP-27 or 50 nM VIP), used as internal positive controls, potently stimulated increases in intracellular calcium (Figure 6), presumably due to action of these peptides on the PACAP-preferring PAC_1 receptor in these cells (cells (Akiho H. et al., *Gastroenterol*, 109: 1105-1112 (1995)).

Natriuretic peptide receptor gene expression in Neuro2a cells

RT-PCR and Northern blot analysis were used to detect the three natriuretic receptor genes in Neuro2a neuroblastoma cells. Northern blot analysis with 15 μg polyA-selected RNA from Neuro 2a cells confirmed expression of NPRA and NPRB genes, but failed to visualize the expression of NPRC gene in Neuro2a cells (Figure 7). Internal positive

controls (kidney, spleen and brain RNA) confirmed specificity of hybridizations.

Because the pharmacological data strongly suggested the presence of NPRC in Neuro2a cells, detection of natriuretic peptide receptor gene expression was attempted using a more sensitive RT-PCR procedure. Primers corresponding to sequences on the cloned receptor (Genbank accession number D78175) were used in the PCR reaction (Figure 8A). Specific amplified bands were detected on Southern blots using ^{32}P-labelled oligonucleotide probes, which corresponded to sequence between the PCR primers. In this analysis, hybridizing NPRA and NPRB bands of the expected sizes were obtained from Neuro2a cell RNA, and were sequenced to confirm their identity. However, no band could be amplified from mouse Neuro2a cells using the NPRC primers. In parallel experiments, the same primers amplified the NPRC message in positive control kidney RNA. Further analysis using multiple primer pairs corresponding to sequences spanning the NPRC mRNA failed to reveal the presence of NPRC in Neuro2a cells, but in all cases amplified the NPRC message in mouse embryonic neural tube (Figure 8B).

The data described in this Example indicate that natriuretic peptides regulate the proliferation of mouse Neuro2a neuroblastoma cells via specific receptor subtypes and signaling pathways. First, ^{3}H-thymidine incorporation was conducted over a 5 hour-treatment period with the different ANP analogs to determine if these cells expressed functional natriuretic receptors capable of regulating growth. Low concentrations of ANP and CNP were found to cause a slight increase in DNA synthesis that was sensitive to PKG blockade, indicative of action on NPRA and/or NPRB (GC) ("type C") natriuretic receptors. On the other hand, low concentrations of the NPRC specific agonist desANP$_{4-23}$, and high concentrations of ANP and CNP, inhibited proliferation. Because low concentrations of desANP$_{4-23}$ and higher concentrations of ANP and CNP can act on NPRC, it seemed likely that the antiproliferative actions were mediated by a type C receptor. This possibility was reinforced by the finding that HS142-1, which specifically binds to and inhibits NPRA and NPRB, had no effect on the growth-inhibitory effects of these natriuretic peptides. Further, radioligand binding studies demonstrated that approximately one third of the total ^{125}I-ANP

binding sites were insensitive to HS142-1, and that desANP$_{4-23}$ displaced ^{125}I-ANP binding completely, and with high affinity. In addition, the action of desANP$_{4-23}$, like that of PHI, was sensitive to inhibition of MEK1/2, indicating that both of these peptides inhibit growth via an ANP signaling pathway (Prins et al., *J. Biol. Chem.* 271:14156-14162 (1996)). Radioligand binding studies also demonstrated the ability of PHI to displace the ^{125}I-ANP binding.

In light of the pharmacological data implicating the existence of a clearance receptor, the inability to detect NPRC gene expression in Neuro2a cells was an unexpected discovery. The Northern analysis was carried out with polyA-selected RNA from approximately 10^8 Neuro 2a cells. RT-PCR analyses with primers corresponding to the mouse NPRC amplified the receptor cDNA only in RNA from adult mouse kidney, a known site of NPRC expression, as well as the E10.5 mouse neural tube. In addition, RT-PCR experiments were repeated using different sets of consensus primers designed to amplify clearance receptor cDNAs from various species. These primers failed to amplify either a normal or variant form of the clearance receptor from Neuro2a cell RNA, but in all cases amplified the receptor cDNA form E10.5 mouse neural tube (Figure 8).

This evidence suggests a novel "clearance-like" receptor, NNPR that interacts with desANP$_{4-23}$ and mediates the growth-inhibitory actions of natriuretic peptides and PHI-related neuropeptides. Further, the fact that ^{125}I-ANP, -PACAP and -PHI binding to Neuro2a cells was displaced by VIP and natriuretic peptide analogues demonstrates that ANP binding sites were present which also interacted with PHI, VIP and PACAP. The binding data obtained suggest that PHI and perhaps VIP and PACAP sites are located on ANP receptors, or that ANP analogs bind to VIP or PACAP receptors. The fact that both desANP$_{4-23}$ and PHI anti-proliferative action were blocked by the MEK1/2 inhibitor PD98059, suggests that PHI and desANP$_{4-23}$ act on the same MAP kinase pathway. A possible model for the effects of VIP, PACAP, PHI and natriuretic peptides such as ANP, on proliferation is depicted in Figure 9.

EXAMPLE II

This example describes the effects of natriuretic peptides on the proliferation of human and rodent neuroblastoma cell lines.

Cell cultures.

Neuro2a neuroblastoma cells were obtained from American Type Tissue Collection (Manassas, VA). SK-N-SH neuroblastoma subclones SHIN and SY-5Y were obtained from Dr. June Biedler (Sloan Kettering Cancer Institute, Rye, NY). NG108 neuroblastoma/glioma hybrid cells were obtained from Dr. Chris Evans, UCLA). All cell lines were maintained in 75 cm^2 flasks (Falcon) in 20 ml of Dulbecco's Modified Eagle Medium (DMEM) (Cellbio/Fisher). Medium was supplemented with 8% fetal bovine serum (FBS) (Gibco-BRL/Life Technology, Gaithursburg, MD) and antibiotics (penicillin/streptomycin) under a 5% CO_2/ 95% air controlled atmosphere at 37°C. Medium was changed every 3 days. Routine subculture of cells was performed by trypsinisation (0.05% trypsin /0.53 mM EDTA, Gibco-BRL/Life Technology Gaithursburg, MD).

Proliferation studies.

Cells were seeded in 24-well plates (80,000 cells/well) in 1 ml medium and cultured for 24 h. Medium was then replaced with fresh serum-free medium. After 1.5 h at 37°C, cells were preincubated for an additional hour with vehicle or signal transduction inhibitors (GF109203, H89 and Rp-8-pCTP-cGMPS from Calbiochem, and PD98059 from New England Biolabs), or the NPR-A/NPR-B antagonist HS-142-1 (provided by Kyowa Hakko Kogyo Co, Shizuoka, Japan). Peptides ($desANP_{4-23}$, ANP and CNP from Sigma, St. Louis, MO) were then added, and then ^{3}H-thymidine (1 uCi/well) was added 1h later. Four hours later, cells were harvested and ^{3}H-thymidine incorporation determined as previously described (Lelièvre V, et al., (1998), *J Biol Chem*, 273: 19685-19690).

Cyclic AMP and GMP measurements

Cells (80,000/well) were cultured for 3 days in 24-well plates. Medium was replaced with serum-free medium with or without 10 mM isobutylmethylxanthine (IBMX) or 10 μM forskolin (Sigma, St. Louis, MO). After incubation for 15 min at 37°C, peptides were added and incubated further for 15 min at 37°C. Cells were lysed in 6% TCA solution and radioimmunoassays were performed following recommendations of the manufacturer (Dupont-NEN).

Intracellular calcium fluorometry

Cells were cultured for three days on glass coverslips until 80% confluence was reached. Cells were then incubated in culture medium for 2h at room temperature in the presence of 5 μM fura-2/AM. Cells were rinsed twice in HBSS medium containing 0.1%BSA, $MgSO_4$, $CaCl_2$, $NaHCO_3$ and $MgCl_2$ (Gibco-BRL / Life Technology, Gaithursburg, MD). Intracellular calcium measurement was performed at 30°C for 300 s on a Hitachi F2000 fluorescence spectrophotometer. Twenty microliters of specified peptides and/or calibration reagents (15ul of 5 mM digitonin and 50ul of 0.2 mM EGTA/pH 8.5) were added into the stimulation buffer (2ml) after 5 min equilibration. The intensity of fluorescence at 340 and 380 nm was converted into calcium concentration. Pituitary adenylyl cyclase activating peptides (PACAP27 and PACAP38) and vasoactive intestinal peptide (VIP) were obtained from Sigma.

Binding studies

^{125}I-ANP (2200 Ci/mmol) was obtained from Dupont/NEN. Binding experiments were conducted on suspended cells in eppendorf tubes (800,000 cells/ml) in binding medium containing 35,000 cpm of ^{125}I-ANP and the specified concentrations of competitive analogs. Incubation was performed at 4°C under gentle agitation. Rinses were performed

and bound radioactivity measurements determined as previously described (Lelièvre V, et al., (1998a), *J Biol Chem*, 273: 19685-19690).

Purification of mRNA

Neuro2a cells were cultured in 8 flasks (75 cm^2) and harvested at 90 % confluency with 0.05 % trypsin/0.02 % EDTA. Trypsin was neutralized with the tissue culture medium (containing 8% serum), cells were centrifuged and then rinsed with phosphate-buffered saline (PBS). Subsequent isolation of polyA-selected mRNA was carried out as described (Lelièvre V, et al., (1998a), *J Biol Chem*, 273: 19685-19690).

Northern and RT-PCR analyses: Total RNA from mouse brain and polyA-selected mRNA from mouse Neuro2a neuroblastoma cells, kidney and spleen tissues were loaded (15 µg/lane) on a 0.8% agarose 2% formaldehyde MOPS gel (Lelièvre V, et al., (1998a), *J Biol Chem*, 273: 19685-19690; Waschek JA, et al., (1998) *Proc Natl Acad Sci USA*, 95: 9602-9607). After transfer to nylon membranes (Magna, MSI), blots were prehybridized overnight and then hybridized as described (Lelièvre V, et al., (1998a), *J Biol Chem*, 273: 19685-19690) for eight hours at 45°C. Natriuretic receptor cDNAs were obtained by RT-PCR experiments performed on mouse kidney RNA. RT-PCR-generated products were cloned into PCR-II vector (InVitrogen) and sequenced to confirm identity. EcoRI-excised cDNA inserts were labeled by random incorporation of ^{32}P-dCTP (Random primers kit, Gibco-BRL, Gaithursburg, MD). After three washes for 20 min at 54°C in 0.2X SSC buffer containing 0.1 % SDS, blots were exposed for three days and signals detected with a Phosphorimager (Molecular Dynamics).

Results

Action of ANP analogs on proliferation

Assay of thymidine incorporation during the four final hours of a five-hour period of peptide treatment revealed that natriuretic peptides modulated proliferation in all neuroblastoma cell lines tested. Natriuretic peptides stimulated NG108 growth dose-dependently (Figure 10A). Proliferation was significantly stimulated by ANP at 0.1 nM, and reached a near maximum 33% increase in proliferation at 10 nM. CNP also stimulated the growth of these cells, although less potently. In contrast, the NPR-C-selective analog desANP$_{4-23}$ (Maack T, et al., (1987) *Science*, 238: 675-678) had no effect. In all other neuroblastoma cell lines, a biphasic action of ANP and CNP was observed (Figure 10B- D). ANP and CNP slightly but significantly stimulated proliferation in all of these cell lines at subnanomolar concentrations, but inhibited cell proliferation at higher doses. Treatment with desANP$_{4-23}$ did not stimulate proliferation at any concentration but inhibited growth beginning at 0.1 nM. Stimulatory and inhibitory effects of natriuretic peptides were unaffected by O-phenanthrolin and bacitracin, which have been shown to potently inhibit endopeptidase activities that cleave natriuretic peptides in other neuroblastoma cells (Delporte C, et al., (1992). *Eur J Pharmacol*, 227:247-56). This indicates that the actions of natriuretic analogs were not likely due to degradation products of natriuretic peptides.

To obtain information regarding the signaling pathways used by natriuretic peptides to control cell proliferation, peptide actions were studied in the presence and absence of various protein kinase (PK) inhibitors. NG108 and Neuro2a cells were selected for these studies. To determine the role of cGMP signaling, cells were pretreated with Rp-8-pCTP-cGMPS (20 uM), a protein kinase G (PKG) inhibitor (Zhuo M, et al., (1994), *Nature*, 368: 635-639). Cells were stimulated for 5 hr with increasing concentrations of natriuretic peptides in the presence of the protein kinase G inhibitor Rp-8-pCTP-cGMPS (15 μM). The small insert at the bottom gives peptide bar codes. Effects of natriuretic

peptides in the absence of Rp-8-pCTP-cGMPS are shown in Figure 10. Stimulatory effects of natriuretic peptides in both cell lines were completely blocked by Rp-8-pCTP-cGMPS (Figure 11A and 11B). In Neuro2a cells, blockade of this stimulation revealed more potent and pronounced growth inhibitory effect of ANP and CNP (Figure 11B vs. Figure 10D).

The MEK1/2 kinase inhibitor PD98059 (20 μM) significantly decreased the basal rate of proliferation of NG108 cells, but did not block the induction of proliferation by ANP or CNP (Figure 12A). The same was true in Neuro2a cells (Figure 12B). Cells were stimulated for 5 hr with increasing concentrations of natriuretic peptides in the presence of MEK1/2 inhibitor PD98059 (30μM). However, PD98059 completely abolished the growth inhibitory actions of higher doses of ANP, CNP and $desANP_{4-23}$ in Neuro2a cells. Thus the antiproliferative actions of natriuretic peptides appear to require the basal activity of a MEK1/2 pathway. Inhibitors of PKA (H89, 20 μM) (Chijiwa T, et al., (1990) *J Biol Chem*, 265, 5267-5272) and PKC (GF109203X, 10 μM) (Toullec D, et al., (1991) *J Biol Chem*, 266, 15771-15781) did not significantly alter the inhibitory actions of any of the natriuretic analogs.

One possible explanation for the inhibitory actions of high concentrations of ANP and CNP in SK-N-SH subclones and Neuro2a cells is that NPR-A and/or NPR-B switch to MAP kinase inhibition and decreased proliferation when presented with higher concentrations of ligands. To determine if inhibitory actions were mediated by NPR-A or NPR-B, DNA synthesis was determined in Neuro2a cells incubated with 10 nM and 1 μM natriuretic peptides in the presence or absence of the NPR-A/NPR-B-selective antagonist HS-142-1 (Matsuda Y (1997) *Contemporary Endocrinology: natriuretic peptides in health and disease* (Samson WK and Levin ER eds) chapt. 17, pp. 289-307, Humana Press Inc, Totowa NJ) (Figure 13). The anti-proliferative actions of ANP, CNP, and $desANP_{4-23}$ were all unaffected by this drug, indicating that the growth-inhibitory effects were probably not mediated by NPR-A or NPR-B. HS-142-1, by itself, had only a negligible effect on DNA synthesis in untreated cells.

Coupling to cyclic nucleotide messengers and intracellular calcium

Changes in intracellular levels of various second messengers were examined in Neuro2a cells to understand the dual actions of natriuretic peptides on proliferation. Changes in cGMP levels were measured the presence of 10 μM IBMX to inhibit phosphodiesterase activity. ANP and CNP potently triggered an approximate 6-fold increase of cGMP levels in Neuro2a cells, whereas $desANP_{4-23}$ did not produce any measurable change (Figure 14). These data corroborated the pharmacological results, which showed that the growth-stimulatory actions of ANP and CNP were mediated by a cGMP/PKG pathway, presumably via NPR-A and/or NPR-B. On the other hand, NPR-C (the only known target for $desANP_{4-23}$) has been shown in some systems to couple to adenylyl cyclase inhibition and calcium influx (Hirata M, et al., (1989) *Biochim Biophys Acta*, 1010: 346-351; Maack T, et al., (1987) *Science*, 238: 675-678). Thus peptide-induced changes in cAMP levels were measured in the presence of 10 μM forskolin. None of the ANP analogs affected cAMP levels under these conditions. Changes in intracellular calcium levels were measured in Neuro2a cells grown on glass coverslips, in response to 0.1, 10 or 20 nM of natriuretic peptides ANP, CNP, and $desANP_{4-23}$. None of the natriuretic peptides induced changes in intracellular calcium levels (Figure 15). Calcium movements induced by the indicated peptides were recorded for 250 sec, before addition of the calibration reagents. In contrast, other neuropeptides (1 nM PACAP-38, 10nM PACAP-27 or 20 nM VIP) used as internal positive controls, stimulated increases in intracellular calcium, presumable due to action of these peptides on the PACAP-preferring PAC_1 receptor in these cells (Lelièvre V, et al., (1998a), *J Biol Chem*, 273: 19685-19690).

Pharmacological characterization of natriuretic receptors

^{125}I-ANP binding was determined on NG108 and Neuro2a cells in the presence and absence of increasing concentrations of ANP, CNP and ANP_{4-23}. For NG108 cells,

displacement curves (Figure 16A) and derived IC50s (Table II) indicated that natriuretic peptides displaced ^{125}I-ANP with the following potency: ANP>CNP>desANP$_{4-23}$. Suspended cells were incubated 4°C for 150 min. Data displayed were the mean ± SEM of 2 independent experiments each performed in triplicate. Graphs and curve fittings were computerized using Graphprism™ software. Hill values were near unity for all analogs, suggesting ligand/receptor interactions occurred on an apparent single ^{125}I-ANP binding site. The observed analog rank potency suggests that the receptor on NG108 cells is NPR-A (Nakao K, et al., (1992) *J Hypertension*, 10: 1111-1114).

Table II.

	^{125}I-ANP displacement in NG108		^{125}I-ANP displacement in Neuro2a	
Ligands	IC$_{50}$±SEM (nM)	Hill slope (±SEM)	IC$_{50}$±SEM (nM)	Hill slope (±SEM)
ANP	0.2±0.074	0.95±0.03	4.26±0.06	0.62±0.08
CNP	2.4±0.14	0.91±0.01	0.24±0.04	0.54±0.2
desANP$_{4-23}$	127±1.2	0.89±0.04	0.30±0.06	0.49±0.4

Table II shows the displacement of ANP radioligand by unlabelled analogs in Neuro2a and NG108 cells. Displacement parameters were extracted from curves given in Figure 16.

In Neuro2a cells, displacement curves revealed a more complex situation. Both CNP and desANP$_{4-23}$ were more potent than unlabeled ANP in displacing ^{125}I-ANP (Figure 16B). Using a one-site competition equation to graph the displacement curves, Hill values obtained were below 0.7 (Table II). This suggested that natriuretic peptides interact on Neuro2a cells through multiple binding sites. This is more apparent in Figure 17A, which

shows the full set of data points for displacement by CNP and desANP$_{4-23}$ (fewer data points were available for ANP displacement; these are all shown in Figure 16B). Using a partial F-test to compare one vs. two site models (Lelièvre V, et al., (1998b). *Eur J Pharmacol*, 341: 299-308), it was found that CNP and desANP$_{4-23}$ displacement curves were statistically better described using a two-site competitive binding equation (Figure 17A). Using this model, displacement of radiolabeled ANP by CNP revealed high affinity (IC50: 74±2.06 pM) and low affinity (8.1±0.45 nM) sites. The high affinity sites represented about 70±0.6 % of the total binding. The opposite situation was observed using desANP$_{4-23}$ as displacing agent. Approximately 35 % of the total binding sites were high affinity (IC_{50}: 9±0.26 pM) whereas the remaining 65% were low affinity (IC_{50}: 1.5±0.18 nM). Taken together these data suggest that two distinguishable binding sites are present in Neuro2a cells.

To further analyze the nature of the binding sites on Neuro2a cells, displacement of ^{125}I-ANP with the NPRA/NPRB antagonist HS142-1 was performed. This compound was able to maximally inhibit about 65% of ^{125}I-ANP binding, with an IC_{50} of about 7 μg/ml (Figure 17B). This suggested that about 65% of the binding was due to interaction of ^{125}I-ANP with NPR-A and/or NPR-B, while the remaining 35% was due to interaction with another site. To determine if the residual HS142-1-insensitive ^{125}ANP binding sites could be displaced by desANP$_{4-23}$, cells were preincubated with 25 μg/ml HS142-1 (a concentration which maximally inhibited ^{125}I-ANP displacement by unlabeled ANP). Under these conditions, desANP$_{4-23}$ was able to displace ^{125}I-ANP binding with high affinity (IC_{50} of about 22±3.5 pM (Figure 17C)). The amount of ^{125}I-ANP displaced (about 200 cpm) was equivalent to about 35% of the total specific binding in the absence of HS142-1 that is displaced by desANP$_{4-23}$ with an high affinity (Figure 17A).

Natriuretic peptide receptor gene expression in Neuro2a cells

Northern blot analysis was used to detect the three known natriuretic receptor mRNAs in Neuro2a cells. Lanes contained 15 μg of polyA-selected RNA, extracted from Neuro-2a,

kidney, spleen and 15μg of total RNA from brain, respectively. Hybridizations were performed overnight at 45C with the corresponding probes as described in Methods. Ribosomal subunits 28 and 18S were used as size markers. Hybridization to polyA-selected RNA revealed expression of NPR-A and NPR-B genes in Neuro 2a cells, but failed to show the presence of an NPR-C mRNA in Neuro2a cells (Figure 18). Internal positive controls (kidney, spleen and brain RNA) confirmed specificity of hybridizations.

Discussion

The data described here indicate that natriuretic peptides regulate the proliferation of neuroblastoma cell lines in a cell-specific manner. In one cell line (NG108), ANP and CNP induced purely a dose-depended increase in thymidine incorporation by a mechanism that involved cGMP-dependent PKG. Growth stimulatory effects were also observed in SK-N-SH subclones and Neuro2a cells at low concentrations of natriuretic peptides. However, at higher peptide concentrations, the growth stimulatory effects in these cells were apparently overridden by a growth-inhibitory mechanism. This inhibitory effect was sensitive to the MEK1/2 inhibitor PD98059.

The dose-dependent stimulation of proliferation was completely blocked by the PKG inhibitor Rp-8-pCTP-cGMP. This suggests the involvement of NPR-A or NPR-B receptors. These contain a GC domain in the intracellular portion of the receptor that is classically activated by agonist binding. The fact that ANP was significantly more potent than CNP in inducing proliferation and displacing ^{125}I-binding in NG108 cells, suggests that the primary receptor subtype expressed was NPR-A. Natriuretic peptide stimulation of proliferation via cGMP induction has been observed in other cell types, for example chick embryonic cardiomyocytes (Koide M, et al., (1996) *Differentiation*, 61: 1-11). The GC pathway also appears to mediate the stimulatory effects of natriuretic peptides on bone growth (Yasoda A, et al., (1998) *J. Biol Chem*, 273: 11695-11700).

The actions of natriuretic peptides on the growth SK-N-SH subclones and Neuro 2a cells were more complex. Low concentrations of ANP and CNP induced an increase in DNA synthesis that was sensitive to PKG blockade, again indicative of action on NPR-A and/or NPR-B (GC) natriuretic receptors. On the other hand higher concentrations of ANP and CNP inhibited proliferation in a manner that was insensitive to PKG blockade. This inhibitory action was fully abolished when cells were also incubated with the MEK1/2 inhibitor PD98059. This may imply that natriuretic peptides act downstream of a growth-stimulatory MEK1/2 pathway that is constitutively-active in these cells under the study conditions. Alternatively, it is possible that growth-inhibitory concentrations of natriuretic peptides act via MEK1/2 induction.

The data reported in this example clearly implicate GC receptors (most likely NPR-A and/or NPR-B) in the growth stimulatory actions of natriuretic peptides in neuroblastoma cells. To support this contention, gene expression of both NPR-A and NPR-B mRNA was confirmed in Neuro2a cells by Northern analysis. In addition, radioligand displacement experiments in both NG108 and Neuro2a cells confirmed the existence of high affinity ^{125}I-ANP binding sites consistent with NPR-A and/or NPR-B. The identity of receptor that mediated the growth inhibitor effects is not certain. One hypothesis is that NPR-A and/or NPR-B receptors are coupled to both stimulatory and inhibitory actions. For example, in the presence of higher concentrations of natriuretic peptides, these receptors might switch to a non-GC growth-inhibitory signaling pathway. This possibility was tested by treating cells with the NPR-A/NPR-B-specific inhibitor HS142-1. This reagent did not prevent the growth-inhibitory effects, suggesting that neither NPR-A nor NPR-B was involved in the antiproliferative actions. An alternative possibility was that the growth inhibitory actions are mediated by another receptor. To support this possibility, radioligand-binding studies indicated the existence of both HS142-1-sensitive and HS142-1-insensitive ^{125}I-ANP binding sites.

One receptor that could mediate the inhibitory action of natriuretic peptides on neuroblastoma cells is the type C "clearance" receptor (NPR-C). It has been proposed by

that NPR-C might have a signaling function (reviewed by Anand-Srivastava MB and Trachte GT (1994) *Pharmacol Rev*, 45: 455-497). This was based on the fact the ligands reported to be specific for NPR-C, for example desANP$_{4-23}$, could regulate the proliferation of some cells and certain other actions by increasing intracellular calcium levels, adenylyl cyclase inhibition, or decreased MAP kinase activity. Thus it was of interest to include desANP$_{4-23}$ as a ligand in the experiments. Interestingly, relatively low concentrations of desANP$_{4-23}$ selectively mimicked the MEK1/2-sensitive antiproliferative actions of high concentrations of ANP and CNP in SK-N-SH subclones and Neuro 2a cells. This suggested that desANP$_{4-23}$ and higher concentrations of natriuretic peptides might act via a common receptor, most likely NPR-C. That a non-GC receptor was expressed in Neuro2a cells was supported by the fact that the HS142-1-insensitive ^{125}I-ANP binding sites could be completely and efficiently displaced by desANP$_{4-23}$ (Figure 17C). However, Northern analysis revealed gene expression for NPR-A and NPR-B only, leaving unclear the molecular nature of the receptor that mediates the growth inhibitory actions of the natriuretic peptides. Among the possibilities: 1) NPR-C is expressed in Neuro2a cells, but at levels below detection by Northern analysis, and 2) a receptor other than NPR-C that binds both natural natriuretic peptides and desANP$_{4-23}$ mediates the growth inhibitory actions. Interestingly, a normal NPR-C as well as an NPR-C-like receptor (termed "type D") was recently cloned from eel. Curiously, expression of the type D receptor in COS cells confirmed its high affinity for desANP$_{4-23}$, but revealed an unexpected sensitivity to HS141-2 (Matsuda Y (1997) *Contemporary Endocrinology: natriuretic peptides in health and disease* (Samson WK and Levin ER eds) chapt. 17, pp. 289-307, Humana Press Inc, Totowa NJ), an inhibitor of the GC-natriuretic peptide receptors NPR-A and NPR-B. Other groups have proposed the existence of atypical "NPR-C-like" receptors in rats (Brown J and Zuo Z (1995) *Am J Physiol*, 269: R261-R273). The data obtained here with a neuroblastoma cell line suggests that a natriuretic peptide ligand/receptor system functions in embryonic nervous system development.

The data also suggests potential proliferative and antiproliferative actions of natriuretic peptides on neuroblastoma tumor growth. If the opposing actions of natriuretic peptides

on neuroblastoma cell proliferation are mediated by different receptors, then the balance of these receptors (i.e. the relative amounts of and types of receptors on each cell) might influence the overall growth of these cells in response to endogenous natriuretic peptides. For example, in a method of therapy, the amount and/or activity of NPR-A receptors might be increased while decreasing the amount and/or activity of NPR-B receptors such as by using antisense oligonucleotides complementary to a NPR-A receptor or NPR-B receptor to inhibit the expression of the receptor,. Alternatively, ligands that specifically antagonize NPR-A and/or NPR-B receptors, or that stimulate non-GC growth receptors involved in growth inhibition can be used to alter the balance and thus activity of these receptors.

EXAMPLE III

This example describes that gene expression, for natriuretic peptides and their receptors, was detected in the discrete regions of the embryonic brain and developing blood vessels at the onset of neurogenesis.

Although it was proposed, almost ten years ago, that natriuretic peptides play important roles in the development of certain organ systems, the recent reports of natriuretic transgenic and knockout mice exhibiting severe skeletal defects have added strong evidence that natriuretic peptides can act in growth-related capacities. With respect to the development of the brain, there is ample data indicating that high affinity natriuretic binding sites are expressed in the early developing brain and associated blood vessels. Further, natriuretic peptides have been shown to potently regulate the proliferation of cultured neuronal and glial cells, and regulate the proliferation and recruitment of blood vessel cell components.

For several years, mechanisms regulating the expression of neuropeptides and receptors during development and the role of neuropeptides in the developing nervous system and nerve regeneration have been examined. Recently, the developmental roles of a

neuropeptide called pituitary adenylate cyclase activating peptide (PACAP) were examined. Gene expression for PACAP and one of its receptors (a seven-transmembrane G protein-coupled protein) were determined to be widely expressed in the embryonic mouse neural tube before closure (Waschek JA, et al., (1998) *Proc Natl Acad Sci USA*, 95:9602-7). Further, *in vitro* evidence demonstrated that PACAP regulates neuroblast proliferation and antagonizes *sonic hedgehog* (*shh*) signaling to regulate dorsal/ventral patterning (Waschek JA, et al., (1998) *Proc Natl Acad Sci USA*, 95:9602-7). Currently, this hypothesis can be tested *in vivo* by creating knockout mice and utilizing the Xenopus embryo mRNA injection system. In related work, signal transduction pathways used by PACAP to regulate neuroblast growth using a neuroblastoma tumor cell line model were examined (Lelièvre V, et al., (1998) *J Biol Chem*, 273: 19685-19690). It was reported that PACAP-related peptides might in certain cases act on receptors that classically mediate the action of a seemingly unrelated family of peptides, the natriuretic factors (Murthy KS, et al., (1998) *Am J Physiol*, 275:C1409-16). Because the proliferative action of PACAP and related peptides in neuroblastoma cells could not be fully explained by the presence of known PACAP receptors, the expression of natriuretic receptors coupled to proliferation was examined. Neuroblastoma cells were found to express functional NPRAs and NPRBs, which were positively coupled to growth via guanylate cyclase. NPRC or NPRC-like receptors were also present, but were negatively coupled to growth via MAP-kinase inhibition.

The growth-related actions of natriuretic peptides in neuroblastoma cells questioned whether these peptides might also have actions relevant to the developing nervous system. Subsequent findings revealed distinct patterns of natriuretic peptide and receptor gene expression in the embryonic mouse nervous system, and have led to the proposal that natriuretic peptides have important roles in nervous system development.

Embryonic expression of natriuretic peptides and their receptors in the nervous system and associated blood vessels

A set of experiments was carried out to determine the temporal and spacial pattern of gene expression for natriuretic peptides and receptors in mid-gestation mouse embryos using *in situ* hybridization. *In situ* hybridization was performed on transverse sections of mouse embryos using ^{32}P-labelled riboprobes corresponding to the known natriuretic peptide receptors (types A, B and C) and autoradiographic procedures. Patterns of expression of these receptors was compared to those obtained previously using receptor autoradiography (Brown et al., *Am. J. Physiol.* 269:R261-73 (1995); Zorad et al., *Eur. J. Pharmacol.* 241:195-200 (1993); and Scott et al., *Anal. Embryol. (Ber.)* 183:245-249 (1991)).

Experiments were conducted on mice at embryonic day (E) 10.5, and at E12.5 (approximately equivalent to E11.5 and E13.5 in rats, respectively, and E0.5 is the morning after fertilization) using *in situ* hybridization. At E10.5 in mice, the central nervous system consisted primarily of a layer of proliferating cells (folded into a tube, but not fully closed at the anterior end). At this stage, vascular invasion of the CNS has just begun (Herken R, et al., (1989) *J Anat*, 164:85-92; Bauer HC, et al., (1993) *Brain Res Dev Brain Res.*, 75:269-78; Conradi NG and P Sourander (1980) *Acta Neuropathol (Berl)*, 50:221-6). The E12.5 time point was selected because a previous report indicated that CNP might be present in the rat brain at the equivalent stage (Cameron VA, et al., (1996) *Endocrinology*, 137:817-24) and also because glial cells and glial cell precursors are more apparent at this stage. The following sections use the terminology established by the Boulder Committee (The Boulder Committee (1970) *Anat Rec*, 166:257-61) to describe the zones of the embryonic CNS.

NPRC gene expression

At E10.5, NPRC gene expression (using a ^{33}P-labeled mouse NPRC riboprobe (Waschek JA, et al., (1998) *Proc Natl Acad Sci USA*, 95:9602-7)) could clearly be detected in the area surrounding the neural tube, presumably the perineural vascular plexus (Figure 19A), major arteries (Figure 19 B, C, and D) and to a lesser extent in veins. Expression was also observed in the notochord (Figure 19 C and D), a structure well known to produce factors (such as *sonic hedgehog*) responsible for dorsal/ventral pattering of neurons and glia in the neural tube. Expression was also observed in the heart and surface ectoderm. Within the nervous system, expression was detected in (or near) the lamina terminalis (Figure 19E), in several cranial ganglia, for example, the trigeminal ganglia (Figure 19F). The expression was presumed to be in satellite cells rather than neuroblasts, which is consistent with receptor autoradiographic findings (Scott, J.N. and Jennes, L. (1991), *Anat Embryol (Berl)* 183: 245-9).

At E12.5, NPRC gene expression continued to be observed in the perineural vascular plexus and surface ectoderm (Figure 20 A and B), and in several peripheral ganglia (dorsal root ganglia shown in Figure 20 C and D; trigeminal ganglia in Figure 20 E and F). Analyses of sagittal sections in the area of the trigeminal ganglia clearly revealed expression in the trigeminal, maxillary, and mandibular nerves, very likely in the Schwann cells (Figure 20 E and F). The expression of NPRC in Schwann and ganglionic satellite cells may be significant in angiogenesis, because glia cells are believed to be key mediators of angiogenesis in neural tissue, expressing VEGF in response to hypoxia (Smith LE, et al. (1994) *Invest Ophthalmol Vis Sci* 35:101-11; Stone J, et al. (1995) *J Neurosci* 15:4738-47; Pierce EA, et al. (1995) *Proc Natl Acad Sci U S A* 92:905-9; Provis JM, et al. (1997) *Exp Eye Res* 65:555-68). The expression in Schwann cells is also significant because these and other glial cells are key mediators for the regeneration of nerves after nerve injury or neurodegeneration.

New sites of CNS gene expression at E12.5 included the roof plate and two bilateral stripes that surrounded the floor plate (Figure 20 G and H), and the choroid plexus. The expression in the choroid plexus suggest that the receptors may be involved in controlling fluid in the brain and may be important in brain edema (for example, after a stroke) and hydrocephalis in fetus and newborn children. Small discrete clusters of NPRC gene transcripts were also observed within the neuroepithelium, presumably over capillaries that had invaded the neural tube (Figure 20 I and J). It was believed that the two bilateral stripes of signals surrounding the floor plate (Figure 20 G and H) represented expression in glial precursors because a similar pattern of expression was observed for two pre-oligodendrocyte markers, PDGFRα-R ((Pringle NP and WD Richardson (1993) *Development*, 117:525-33) and PLP/DM20 (Yu WP, et al., (1994) *Neuron*, 12:1353-62). The expression in oligodendrocytes precursors are relevant as the oligodendrocyte cess are responsible for re-myelinating nerves, and are implicated in certain diseases, such as multiple sclerosis.

NPRB and NPRA gene expression

At E10.5, NPRB was consistently expressed at low levels on cells just outside the VZ of the hindbrain, presumably corresponding to early postmitotic neurons (Figure 21 A and B). Much higher gene expression was observed in many developing peripheral ganglia (trigeminal ganglia in Figure 21 A and B, and dorsal root ganglia in Figure 21 C). No expression was detected in vascular tissue, or in any areas outside of the nervous system. In contrast, no NPRA gene expression was observed within the brain or in any ganglia, while a very low level of expression was observed at E10.5 and E12.5 in blood vessels of the head, and in the surface ectoderm.

Natriuretic peptide (ligand) gene expression

Natriuretic peptide gene expression at E12.5 and for a few sections at E14.5 was examined. Sagittal sections at E12.5 revealed CNP gene transcripts at all levels of the

CNS caudal to the mesencephalon (Figure 22 A and B). Transverse sections at the level of the hindbrain revealed that the CNP transcripts were localized in the VZ at the mid-dorsal region (Figure 22 C and D). ANP and BNP gene transcripts were not detected in the nervous system, but were expressed at very high levels in the heart. At E14, CNP gene transcripts were still observed at high levels in the VZ, although some gene expression was observed in cells in the hindbrain outside the VZ (Figure 22 E and F). Specific hybridization signals were also observed in cells that surrounded the neural tube, most likely in cells associated with the dura layer of the meninges (arrows in Figure 22E and F).

Antiproliferative action of natriuretic peptides on cultured neuroepithelial cells

The above studies showing natriuretic peptide receptor gene expression in the brain suggested that one action of natriuretic peptides might be to regulate some aspect of neurogenesis. Using the methods previously employed, ^{3}H-thymidine incorporation in cells prepared from neural tubes isolated from E10.5 mice was examined (Waschek JA, et al. (1998). *Proc Natl Acad Sci USA* 95:9602-7). Cells were isolated from E10.5 mouse neural tubes and cultured as described (Waschek JA, et al., (1998) *Proc Natl Acad Sci USA*, 95:9602-7). Peptides were added at the indicated concentrations. After 24 hr, ^{3}H-thymidine was added, and cells were harvested for ^{3}H-thymidine incorporation 6 hr later. The effect of BNP was not examined. ANP peptides decreased DNA synthesis in a dose dependent manner (Figure 23), indicating that these peptides exerted an antiproliferative action. The potent action of CNP (which acts efficiently only on NPRB and NPRC) was consistent with *in situ* hybridization studies showing the presence of NPRB gene expression. The fact that the NPRC-specific analog desANP$_{4-23}$ inhibited proliferation indicated that at least some of the antiproliferative actions may be mediated through a NPRC or NPRC-like receptor. This was consistent with the data of others indicating that desANP$_{4-23}$-sensitive binding sites are present in the embryonic brain (Zorad S, et al., (1993) *Eur J Pharmacol*, 241:195-200; Brown J and Z Zuo (1995) *Am J Physiol*, 269:R261-73).

Results of the hybridization using riboprobes showed that when the nervous system consists primarily of a single cell layer of proliferating cells folded into a partially-closed tube (embryonic day or "E" 10.5), type C receptor gene transcripts were detected not only in the lamina terminalis and near the optic stalk, but also in the condensing trigeminal, VII, and VIII ganglia neural crest. The type C receptor gene was also expressed in the notochord, suggesting a role of this receptor in dorsal/ventral patterning of the nervous system. A low level of Type B receptor gene transcript appeared in the few postmitotic neurons just outside of the proliferating cells in the ventricular zone. Type A receptor transcripts were not observed anywhere in the neural tube or developing ganglia. However, abundant type A and C receptor gene transcripts were detected in surrounding blood vessels and in the heart. At E12.5, type C receptor transcripts again were detected in the lamina terminalis, in the trigeminal, VIII, IX and dorsal root ganglia and notochord, but also in the roof plate of the neural tube. The type C receptor gene was also very clearly expressed in narrow stripes in the ventricular zone on each side of the floor plate in the spinal cord. The telencephalon, a region in the rat which has shown a high degree of specific binding with the characteristics of a clearance-like ANP receptor (Brown et al., and Zorad et al., supra), notably lacked known type C receptor gene expression. However, the autoradiography indicated that significant type C-like receptor binding was present in the proliferative zones of the telencephalon, despite the absence of gene expression for known type C receptor.

Prior researchers have found that natriuretic peptide receptor binding sites were present in the juxtaventricular zone of the telencephalon in E14 and E17 rats (Brown et al., and Zorad et al., supra). These sites were characterized by radioligand displacement using several ANP analogues and displayed a type-C like nature (for example, binding was fully displaced by desANP$_{4-23}$). Because the results in this example demonstrated the absence of type C receptor gene expression in this region at approximately the equivalent stage in mice, these results suggest that the binding sites in the telencephalon represent a novel type C-like receptor, NNPR, that mediates the action of desANP$_{4-23}$ and PHI in neuroblastoma cells as set forth above in Example I. Alternatively, these results could represent presence of

another novel receptor that may be involved in the proliferation of brain cell.

In summary, previous findings that natriuretic peptide receptors were expressed in the embryonic nervous system and associated blood vessels were confirmed. Localized receptor gene expression was detected at stages earlier than that previously reported, at E10.5 (near the time of onset of neurogenesis and brain vascularization). The primary receptor subtype on developing blood vessels, Schwann cells, ganglion satellite cells, and presumed oligodendrocyte precursors was found to be NPRC. The subtype on newly differentiating neurons was found to be NPRB. The expression of NPRC in Schwann and ganglionic satellite cells may be significant in brain vascularization, because glia cells are thought to be important for angiogenesis in neural tissue and in the formation of the blood-brain and blood-nerve barriers. The NPRC gene was also shown to be expressed in the roof plate and notochord. These structures play important roles in dorsal/ventral patterning and axonal guidance in the CNS. Gene expression for the ligand CNP was detected at high levels in the VZ in the hindbrain and spinal cord, providing an endogenous source of natriuretic ligand within the developing brain. The proliferation of E10.5 neural tube neuroblasts in culture was found to be potently inhibited by CNP.

Based on the above data and demonstrations by other groups of the role of natriuretic peptides in regulation of proliferation and migration of blood vessels cell components, it was proposed that natriuretic peptides, produced either in the heart or brain, regulate nervous system development. They may do so by acting directly on developing neurons and glia, and by acting on cells involved in the developing brain vasculature, including glia, endothelial cells, pericytes, and VSMCs.

EXAMPLE IV

This example investigates whether natriuretic peptides regulate the growth of normal embryonic neuroblasts during development. Neuroepithelial cells were isolated from mouse E10 embryos as described by Waschek et al., *J. Neurochem.* 66:1762-`765 (1996)).

The effects of PHI and desANP$_{4-23}$ peptides on DNA synthesis in the isolated cells were examined. Maximal inhibition by both peptides was about 60 %. The IC_{50} values were about 1 nM and 20 nM for PHI and desANP$_{4-23}$, respectively. As demonstrated in Examples I and II for neuroblastoma cells, the inhibition was blocked when cells were preincubated with MEK 1/2 kinase inhibitor PD98050.

These results suggest that embryonic neurons, like neuroblastoma cells, respond to natriuretic peptides with a decrease in growth that is sensitive to MAP kinase pathway inhibition.

EXAMPLE V

Effects of natriuretic peptides and neuropeptides on neuron survival

This Example describes the ability of natriuretic peptides such as ANP and PHI-related neuropeptides to act as trophic factors (i.e. act on survival) of motor neurons after facial nerve axotomy. This model is widely recognized for evaluating agent that can prevent motor neuron cell death in vivo. (Price et al., *Ciba Found. Symp.* 196:3-13 (1996); Kreutzberg, *Acta Neurochir. Suppl. (Wien)* 66:103-6 (1996); Raivich et al., Keio. *J. Med.* 45:239-247 (1996); deLapeyriere, *Curr. Opin. Genet. Dev.* 7:642-50 (1997) and Kreutzberg, *Arzneimittelforschung* 45:357-360 (1995)). This model has permitted investigators to study the activity of putative trophic factors in vivo using local delivery of these factors to facial motor neurons. The trophic factors GDNF, NT-3, CNTF, BDNF, NT-4/5, IGF-1 and LIF have all been shown to significantly rescue motor neurons in this model. (Gravel et al., *Nat. Med.* 3:765-770 (1997); Baumgartner and Shine, *J. Neurosci.* 17:6504-6511 (1997); Fernandes et al., *J. Neurosci.* 18:9936-9947 (1998); Baumgartner and Shine, *J. Neurosci. Res.* 54:766-777 (1998); Tan et al., *Cell Transplant* 5:577-587 (1996); Gimenez et al., *J. Neurosci. Res.* 48:281-285 (1997); Yan et al., *J. Neurobiol.* 24:1555-1577 (1993); Yan et al., *Nature* 373:341-344 (1995); Yan et al., *J. Neurosci.* 14:5281-5291 (1994), and Hughes et al, *J. Neurosci. Res.* 36:36:663-671 (1993)).

Facial nerve axotomy is performed in neonatal rats as described in the literature. In neonatal rats, facial nerve axotomy results in massive motor neuron death (greater than 80%) within four days. Briefly, animals are anesthetized, the right facial nerve is exposed and a cut is made at the point where the nerve exits from the stylomastoid foramen. The left (contralateral) side serves as a control. In some of these animals, sham operations are also performed on the right side. Other controls include right side sham-operated animals and normal animals. Animals are axotomized at three ages, postnatal day 1, postnatal day 5 and adult (10 weeks). Changes in the predicted responses in the facial motor nucleus (microglia activation, chromatolysis, etc) are observed after administration of VIP, PHI, and ANP cDNAs (in the sense or antisense orientation as appropriate).

Peptides such as VIP, PHI and ANP are delivered to facial motor neurons in Wistar rats and/or C57/BL6 mice using a retroviral delivery system to rescue dying motor neurons and to block the action of endogenous peptides using receptor antagonists. The peptides are delivered to axotomized facial motor neurons by injecting the virus into the rat facial muscle, for retrograde transportation to the facial motor neurons. The encoded gene products are expressed relatively specifically in the facial motor neurons with minimal or no perturbation of either the injury site or the facial motor nucleus. A two-plasmid system is used to generate recombinant adenoviruses containing the VIP, PHI or ANP cDNA (Baumgartner and Shine, *J. Neurosci.* 17:6504-6511 (1997); Baumgartner and Shine, *J. Neurosci. Res.* 54:766-777 (1998)). The first plasmid, pJM17, contains the viral genome with a pBR322 variant (pBRX) inserted into the E1a region. This permits propagation in *E. coli* and prevents formation of active virus producing a first generation vector. The second plasmid is the shuttle vector into which the mouse VIP, PHI or ANP cDNA is inserted. The two plasmids are co-transfected into 293 cells, which are then covered with a mixture of medium and agarose. A homologous recombination event occurs between the two plasmids that result in the replacement of pBRX with VIP, PHI or ANP cDNA. The recombinant vectors that result produce plaques on the culture plates. Plaques are picked and amplified for screening by PCR and large-scale preparation as described by Baumgartner and Shine,

1997 and 1998, supra. The cDNA can be modified to optimize expression in such delivery systems. For example, the cDNA can be engineered to contain an optimal Kozak consensus sequence to increase expression.

Recombinant virus is injected into the rat facial muscle on postnatal day 1 rats. As a control, the parent virus containing the β-galactosidase gene is infected. The gene has been shown to be expressed in facial motor and trigeminal neurons after injection into the facial muscle (Baumgartner and Shine, 1997, supra). Expression of VIP, PHI or ANP and ß-galactosidase is monitored using immunohistochemistry and enzymatic histochemistry, respectively, using standard procedures.

The degree of axotomy-induced cell death after injection of virus is determined. On postnatal day 1 (24 hrs before axotomy), viral vectors containing cDNAs are injected into the left facial muscles of the cheek, lower lip and whisker pad on the side to be lesioned. Animals are sacrificed 48 and 96 hrs after surgery. Rats are anesthetized and perfused with 4% formaldehyde. The brain stem is dissected, post fixed, cryoprotected and sectioned across the region containing the facial motor nucleus. The degree of motor neuron death is assessed on ipsilateral and contralateral sides of the facial motor nucleus on every fifth section using cresyl violet staining. Only motor neurons with a clearly identifiable nucleus and nucleolus are counted. In between sections are used to confirm expression of recombinant proteins.

Data are obtained indicating whether VIP, PHI and/or ANP delay motor neuron death in axotomized animals. Moreover, these factors may enhance efficacy of classical neurotrophins by delaying neuronal death and enhancing *trk* expression.

Alternatively, the effects of VIP, PHI or ANP on neurons in the adult rat are examined. Although motor neuron cell death is minimal in adult animals after axotomy, chromatolysis and decreased choline acetyl transferase (ChAT) immunoreactivity are rapidly observed (Fernandes et al., supra, and Guntinas-Lichius et al., *Cell Tissue Res.* 286:537-541 (1996)).

VIP/PHI/ANP are delivered to motor neurons in axotomized adult rats using the delivery methods described in this Example, and the effects on chromatolysis or ChAT expression are determined. Alternatively, adult BalbC mice are used because axotomy causes greater than 30% neuron cell death after one week in these animals (Ferri et al., *J. Neurobiol.* 34:1-9 1998)).

The ability of the VIP, PHI or ANP to block the death of CA1 neurons in the hippocampus in another injury model, transient forebrain ischemia induced by four vessel occlusion provides another test of the effects of these compounds on neuron survival. (Uchida et al., *Brain Res.* 736:280-286 (1996)). VIP, PHI or ANP is administered by intracerebral ventricular (ICV) route as described by Yan et al., 1994, supra. The peptides are administered, e.g. at 1 pmol/hr, via a subcutaneous miniosmotic pump (Alzet, ALZA) through an inner cannula inserted into a guide cannula implanted into a lateral ventricle as described by Uchida et al., supra. The infusion is begun just prior to the axotomy procedure and continues until the animals are sacrificed.

In still another experiment, VIP, PHI and/or ANP are delivered to axotomized facial neurons using a piece of gelfoam soaked in the factor placed at the lesion site. (de Bilbao and Dubois-Dauphin, *Neuroreport* 7:3051-3054 (1996)).

EXAMPLE VI

This example demonstrates the cloning of a Xenopus natriuretic peptide type-C receptor (XNPR-C) and analysis of its physiological functions. The data provided herein, demonstrates that NPRC is coupled to signaling pathways that are commonly used to regulate cell growth and survival. Further, the action of peptides on NPRC is shown to modify the signaling of unrelated receptors, and thus these actions can affect proliferantion and/or survival indirectly.

Among the numerous actions reported, natriuretic peptides play a key role in maintaining body fluid homeostasis. This action may be crucial for species that experience large environmental variations in salt concentration, for example, fish and amphibians. In the latter, two different isoforms of CNP exist (Kojima M, et al. (1994) *J Biol Chem*, 269:13136-13140) and they may have different functions. Characterization of putative functions of natriuretic peptides in frogs has been initiated. Numerous different species of frog exhibit binding sites for natriuretic peptides in kidney (Kloas W and Hanke W (1992) *Peptides*, 13:297-303; Kloas W and Hanke W (1992) *Gen Comp Endocrinol*, 85:269-277; Kloas W and Hanke W (1993) *Gen Comp Endocrinol*, 91:235-249; Meier SK, et al. (1999) *Gen Comp Endocrinol*, 115:244-253) and in brain (Tong Y, et al. (1989) *J Comp Neurol*, 281:384-396), whereas receptors for ANP and BNP appear to regulate water influx in skin (Uchiyama et al, 1998 *Comp Biochem Physiol C Pharmacol Toxicol Endocrinol* 120:37-42). In addition to these classic effects on water and salt control, ANP-immunoreactivity has been observed in brain and pituitary at early stages of *Rana esculenta* tadpole development, suggesting that natriuretic peptides could play a role during brain and endocrine development (Vallarino M, et al. (1998) *Cell Tissue Res.* 293: 47-55).

METHODS

RT-PCR and isolation of a specific probe

Total RNA was extracted from 2-week-old Xenopus tadpoles. Fifty tadpoles were decapitated, and heads were quickly plunged into cold guanidium isothiocyanate solution prior dissociation using a Polytron homogenizer. Subsequent isolation of total RNA was carried out according to the Chomczynski and Sacchi method (Chomczynski P and Sacchi N (1987) *Anal Biochem* 162:156-159). RNA was reverse-transcribed into cDNA, at 45°C for 30 min using MLVT reverse transcriptase from Perkin Elmer.

PCR primers were designed based on the sequence comparison between mammals (Fuller F, et al. (1988) *J Biol Chem*, 263: 9395-9401; Lowe DG, et al. (1990) *Nucleic Acids Res*, 18:3412; Engel AM, et al. (1994) *J Biol Chem* 269:17005-17008; Yanaka N, et al. (1996) *Eur J Biochem*, 237:25-34) and eel NPR-C (Takashima et al, 1995, *Eur. J. Biochem.* 227, 673-680). Primers sequences were 5'-TGAGGACAGCGAAAC CTGATT-3' and 5'-GCAGGATTCTTCTAGGCCAC-3' for sense and antisense, respectively. This pair of primers (Life technologies, Gaithersburg, MD) was designed to generate fragments of 471, 447, 471 and 462 bp corresponding to nucleotides 1427-1868, 869-1330, 1807-1336, 967-1429 and 945-1399 for NPR-C from bovine, eel, human, mouse and rat, respectively. PCR amplification was carried out for 35 cycles of denaturation (94C, 50s), annealing (48C, 45s and extension (72°C, 45s), using increasing concentrations of $MgCl_2$ from 2 to 4 mM. A last extension at 72°C for 5min concluded the PCR method. A single band of 450bp was observed on a 2% agarose gel at the optimal $MgCl_2$ concentration of 2.5 mM. This amplified product was cloned into PCR2.1 vector using TA cloning kit (Invitrogen, San Diego, CA). Positive clones were detected on X-gal and ampicillin containing plates, and amplified. Digestion of the resulting plasmids using EcoR-I restriction enzyme showed the 450 bp insert corresponding the PCR-amplified product.

In parallel, these gels were transferred overnight onto a nylon membrane (Nylon+, from MSI) and hybridized with a consensus internal primer (5'-GAAGGGWC CGCCGGGCAGGTTC-3') corresponding to the nucleotides 1631-1655, 1076-1100, 1540-1558, 1163-1184 and 1179-1200 of the NPR-C sequences from bovine, eel, human, mouse and rat respectively. This oligoprobe was ^{32}P-end-labelled using gamma-ATP (ICN) and polynucleotide kinase (Promega). Prehybrization (6 hrs) and hybridization (4 hrs) were performed at 37 °C. Membranes were rinsed 3 times (15 min each) in 1X SSC /0.1% SDS buffer at 42 °C. Radioactivity bound was analyzed using phosphorimager (Molecular dynamics) after 2 hours exposure. Strong labeling of the 450 bp insert was observed on both membranes. Sequence analysis of the 450 bp PCR product revealed strong homology of sequence with mammalian and eel NPRC receptors.

Xenopus brain library screening

The Xenopus PCR-generated probe (450 bp) was radiolabelled with [alpha-^{32}P] dCTP random primer labeling kit (Life Technologie, Gaithersburg, MD). This probe was used to screen a *Xenopus* brain lambda Ziplox cDNA library (GibcoBRL, Gaithersburg, MD) provided by Dr. E. deRobertis (UCLA). Approximately 0.6 X10^6 plaques were screened at an initial density of 20,000 plaques/plate (10 cm bacterial plates) and transferred onto nitrocellulose membranes (MSI). Membranes were air-dried for 10 min. Phage plaques were gently lysed in 1.5 M NaCl /0.2M NaOH solution for 2 min. Membranes were carefully neutralized within 2 X SSC/0.4M TRIS (pH 7.4) for 1 min then washed for 5 min within 2X SSC buffer. Membranes were air-dried for 15 min then baked for 45 min at 80C, prior UV-cross-linking. Filters were prehybridized in a 5X SSC buffer containing 50% formamide, 0.1% SDS, 5X Denhardt's solution, 0.05 M sodium phosphate (pH=6.5), 50mg tRNA and 20mg Herring sperm DNA, for 6 hrs at 42°C, then hybridized overnight in fresh buffer containing 400,000 cpm/ml of the radiolabeled probe. Four washes (2 for 5 min each, then 2 for 20 min each) were finally performed at 42°C, in low-stringency 1X SSC / 0.1% SDS buffer. Between washes, membranes were allowed to dry for 2 minutes. The radioactivity bound to membranes was analyzed on Phosphoimager (Molecular Dynamics) after overnight exposure.

Four positive clones were subsequently replated and analyzed using the same procedures after dilution of phage suspensions to obtain final concentrations of 100 plaques/plate. Transfers and hybridization of the filters were performed as previously described, two of the 4 positive clones (# 30 and 31) were found on this screen to be negative.

The remaining 2 strongly positive clones (# 36 and 38) were once again diluted and plated to an approximate density of 15 clones per plate. The plates were then transfered and hybridized as above. Isolated phages from positive plaques were transformed into plasmid using the rapid excision procedures according to the manufacturer (GibcoBRL, Gaithersburg, MD). The two excised BlueScript plasmids were amplified in DH10bZL

cells (GibcoBRL, Gaithersburg, MD). Restriction site analysis revealed insert sizes of 3 and 2.5 kb, respectively. Sequencing of these clones was performed in both directions.

Cap-mRNA transcription

The cDNA encoding XNPRC was isolated using double digestion with BamH1/Cla1, and was inserted into the pCS2$^+$ vector. Plasmids were amplified in Top10 cells (InVitrogen) and linearized using Nsi1 endonuclease to obtain a template for *in vitro* transcription. Using 10 μg of DNA template, capped XNPRC mRNA was transcribed using Sp6 polymerase and a transciption kit from Promega. Capped messenger RNAs (C-mRNA) were purified with DNaseI and subsequent phenol/chloroform extraction. Purified C-mRNA were reconstituted in RNAse-free water (Ambion) and frozen at –20 □C. Preparation of capped Xenopus PAC1 receptor mRNA was as described (Hu Z, et al. (2000) *Endocrinol*, 141: 657-665).

Injection in Xenopus oocytes

Adult *Xenopus* females were anesthetized by immersion into 0.15% tricaine-containing water for 25 min. On ice, abdomens were opened and ovarian lobes were removed. Lobes were rinsed in calcium-free OR-2 buffer (82.5 mM NaCl, 2.5 mM KCl, 1mM $MgCl_2$, 5 mM HEPES). Lobes were dissociated within calcium buffer OR-2 prior incubation within collagenase (412U/ml). Defolliculated oocytes were isolated from ovaries within 25-35 min under strong shaking conditions at room temperature. Isolated oocytes were gradually transferred to SOS buffer (100 mM NaCl, 2 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$ and 5 mM HEPES, pH= 7) and kept at 18°C into low-temperature incubator (REVCO). Medium was replaced daily.

Under stereomicroscope (Zeiss), oocytes were injected with aqueous solution of XNPR-C mRNA using a Nanoliter™ injector attached to a Kite™ micromanipulator (WPI). Injected oocytes were rinsed twice within SOS buffer, and kept for 4 days at 18°C. For

binding assays, about 30ng of capped mRNA were injected per oocytes, whereas, only 15 ng of NPR-C or/and PAC-1 mRNAs were used for the transduction experiments.

Binding assay

Injected oocytes were rinsed for 20 min in binding medium (SOS buffer containing 0.1 % BSA and a protease cocktail including O-phenanthrolin, bacitracin and PMSF). Each sample contained 8 oocytes.

For saturation experiments, samples were incubated in binding buffer containing increasing concentrations of ^{125}I-ANP (2200 Ci/mmol, NEN), in the presence (non specific) or absence (Total) of 10 μM of native ANP for 150 min at 4°C. Oocytes were carefully rinsed twice with 2 ml of cold wash buffer (Binding medium containing 1 % BSA). Oocytes were transferred to 5 ml tubes and the bound radioactivity was quantified using a Gamma-counter (LKB).

For radiodisplacement experiments, oocytes (8/sample) were incubated for 150 min at 4°C, in binding buffer containing ^{125}*I*-ANP (70,000 cpm/sample) and increasing concentrations (from 0 to 1uM) of the specified analogs. Rat and frog analogs were obtained from Sigma (St. Louis, MO) and Phoenix pharmaceuticals, respectively. HS-142-1 was generously provided by Dr Y. Matsuda (Tokyo Research Laboratories, Tokyo, Japan). Washes and radioactivity measurement were performed as indicated above.

cAMP RIA

XNPR-C-injected oocytes (10/sample) were preincubated in SOS medium containing Forskolin (1 or 10 μM) and IBMX (10 mM) was added for 15 min at room temperature, prior to addition of the specified peptides. Peptides were added for another 15 min period. At the end of the incubation, samples were placed on ice and medium was replaced by 500 μl of TCA (6%) solution. Sonication (10 sec/sample) was used to extract cAMP and

samples were dried out under vacuum. Lysates were reconstituted in 500µl of 0.05 M acetate buffer (pH= 5.8) and diluted 40 times before utilization. Cyclic AMP production in stimulated oocytes was assessed using a RIA kit according to the manufacturer recommendations (Dupont-NEN). Double-injected oocytes (10/sample) with C-mRNAs encoding NPRC and PAC1 receptors were preincubated in IBMX alone or with 50nM PACAP-38, prior to addition of the specified peptides. Cyclic AMP isolation and measurements were performed as described above.

Results

Isolation of a full-length cDNA encoding the Xenopus NPR-C receptor and sequence analysis

Using RT-PCR technique and consensus primers, a DNA fragment of 450 bp was amplified and cloned. This DNA fragment displayed a sequence with high homology to published NPR-C sequences and those available in the NCBI database. Using this DNA fragment, a lambda phage cDNA library was prepared and screened. Fifty plates containing about 20,000 plaques/plate were analyzed. Among the four positive plaques observed after the first round of screening, two of them were found to be negative after the second screening. To isolate unique phage clones from the remaining plaques, a last round of screening was performed, where all the plaques strongly hybridized with the radiolabeled probe. Lambda phages were isolated from these plaques, and were transformed into Bluescript plasmid using a Ziplox rapid excision method (GibcoBRL, Gaithersburg, MD).

Restriction fragment analysis of the inserts revealed two clones of 3.0 Kb and 2.6 Kb. Bi-directional sequencing of both clones was performed over the entire sequence. Only the 3.0 kb clone contained a full-length cDNA (Figure 24), whereas the other was highly homologous but lacked 350 bp in the 5'upstream sequence. The sequences were submitted to the Genbank database (accession number AF231035).

Transformation of the largest open reading frame of clone 36 into its predicted amino acid sequence was performed (Figure 24) to analyze the global hydrophobicity of the putative protein. A hydrophobic plot showed a unique region of hydrophobic residues at C-terminal end of the molecule, compatible with the presence of a unique transmembrane spanning domain. This protein sequence contained 6 cysteine residues in the extracellular part of the molecule, 4 (positions 78, 104, 181 and 229) of which may be involved in internal disulfide bonds. The others (cysteine 440 and 443) are too close, but might be involved in receptor dimerization. In addition, 3 putative sites for N-glycosylation are present. A schematic representation of this receptor was shown in Figure 27.

To address the question of the nature of this cDNA, the sequence of this protein was compared with others in the SwissProt database (BlastN). The result of this analysis showed that the open reading frame (ORF) had a very high homology with NPR-C receptors from various species (Fuller F, et al. (1988) *J Biol Chem*, 263: 9395-9401; Lowe DG, et al. (1990) *Nucleic Acids Res*, 18:3412; Engel AM, et al. (1994) *J Biol Chem* 269:17005-17008; Yanaka N, et al. (1996) *Eur J Biochem*, 237:25-34; Takashima et al, 1995, *Eur. J. Biochem.* 227, 673-680), as shown in Figure 25. Specifically, the nucleotide sequence of XNPR-C presented 84%, 83%, 85%, 84%, 83%, 87% and 86% identity with partial sequences of NPR-C from *Bufo marinus* (453 nt), human (793nt), bovine (674 nt), mouse (629 nt), rat (706nt), and eel (166). This represented 37, 35, 32 and 38% of the entire coding sequence of the natriuretic peptide receptor-C of human, bovine, mouse and rat respectively, but only 9.5% of the eel NPR-C sequence. However, results were much more impressive for the deduced protein sequence. Indeed, XNPR-C had 73, 73, 72, 71, 57% identity to NPR-C of the following species, human, bovine, rat, mouse and eel, respectively. This percentage reached 85% of homology with the human sequence. The highest score, 86% of identity (94% homology), was naturally obtained with the published partial sequence (151 aa) of the frog *Bufo marinus*. Interestingly, two other Xenopus sequences, XGC-1 and XGC-2, showing homology with the protein were found in the database (Figure 26). Their percentages of identity were 27 and 34%,

respectively. However these identity scores were similar to those obtained when the sequence was matched against mammalian NPR-A and NPRB (~ 30-31 %).

Pharmacological studies of the XNPR-C

In initial experiments, defolliculated oocytes were injected with 30 ng of XNPR-C capped mRNA. Four days after injection, oocytes were incubated with increasing concentrations of ^{125}I-ANP in the presence or absence of 10 μM of rat ANP_{1-28}. Receptor saturation curves were obtained by incubating oocytes (8/sample) increasing concentration of ^{125}I-ANP with (Non specific) or without (Total) 10 μM ANP for 150 min at 4°C. Specific binding was determined by subtraction the non-specific from the total binding. Curve fitting and graphs were performed using Graphprism™ software (ISI). A non-linear regression analysis of the resulting saturation curve was used to determine the ligands/receptor interaction parameter KD and Bmax. Experiments wereperformed twice in triplicates. The saturation curve showed specific saturation of expressed binding sites for peptide concentrations over 150 pM (Figure 28). Non linear regression of the specific binding curve (obtained after subtraction of nonspecific binding) showed a maximum binding value (B_{max}) of 57± 2.3 X 10^6 sites/oocytes and K_D of 96± 4.23 pM. In contrast, uninjected oocytes showed no specific binding.

Radioligand displacement studies using various unlabeled ANP analogs showed that expressed NPR-C receptors had very high affinities (< 0.2 nM) for rat (Figure 29A) and frog (Figure 29B) ANP analogs (Table III). Binding displacement curves were obtained by incubating oocytes (8/samples) with 60,000cpm of ^{125}I-ANP for 150 min at 4°C, with increasing concentrations of natriuretic analogs from Rat (A) or Frog (B), of VIP-related neuropeptides (C) or again of HS142.1 (D). Experiments were performed twice in triplicates. Curve fittings and graphs were generated using Graphprism™ software (ISI). This included the rat $desANP_{4-23, which}$ is a selective agonist for the type-C receptors.

TABLE III

	Analogs	IC_{50}
RAT ANALOGS	*ANP1-28*	*0.13± 0.076 nM*
	CNP	*0.1± 0.068 nM*
	Des ANP4-23	*0.18± 0.085 nM*
MICROBIAL INHIBITOR	*HS 142-1*	*NA >>>200Ug/ml*
FROGS ANALOGS	*CNP*	*0.09± M*
	ANP	*0.21± 0.078 nM*
	ANP4-24	*0.11± 0.085 nM*
NEUROPEPTIDES	*VIP*	*0.19± 0.09 nM*
	PHI	*0.38± 0.11 nM*
	PACAP-27	*1.4±0.56 nM*
	PACAP-38	*24.7±2.45 nM*

Table III depicts the displacement of 125I-ANP binding by natriuretic peptides and VIP-related neuropeptide analogs. IC_{50} values for the different analogs tested have been calculated from displacement curves of the 125I-ANP binding in NPR-C overexpressing oocytes. Data were extracted using Graphprism™ software.

A study to displace ^{125}I-ANP with VIP-related neuropeptides (Figure 29 C) was based on the suggestion that VIP-related peptides interact with NPR-C (Murthy KS, Makhlouf GM (1999) *J Biol Chem.* 274:17587-17592). Among the neuropeptides tested, VIP and PHI had relatively high affinity, whereas PACAP-27 showed an IC50 value of about 1 nM (Table III). PACAP-38, which is the highest-affinity agonist for the cloned Xenopus PACAP-preferring receptor (PAC1) (Hu Z, et al. (2000) *Endocrinol*, 141: 657-665), had a relatively low affinity for these sites, approximately 25 nM (Table III).

To determine if ^{125}I-ANP binding was inhibited by HS-142-1 (a specific for NPR-A and NPR-B (Morishita Y, et al. (1991) *Biochem Biophys Res Commun*, 176:949-957) and eel NPR-D (Kashiwagi M, et al. (1995) *Eur J Biochem*, 233:102-109)), displacement assay was performed. Radioligand binding was not displaced by various concentrations of HS-142-1 (0 to 200 μM) (Figure 29D).

Coupling to AC

Defolliculated oocytes were injected as described above for binding assays and incubated for 4 days prior stimulation. Oocytes (10/sample) were preincubated within a solution of 1 mM IBMX and 10 μM FSK for 15 min at room temperature prior addition of the specified peptides. Peptides were incubated for another 15 min period, before cAMP extraction. Cyclic AMP concentrations were measured by RIA. Experiments were conducted in triplicates. Peptide stimulations were performed after preincubation in SOS buffer containing IBMX to inhibit phosphodiesterase activity. In the presence of 10 μM FSK to preactivate adenylyl cyclase, basal levels displayed a 12-fold increase when compared to control oocytes incubated in the presence of IBMX alone. Pre-stimulation was followed by a 15 min-incubation with or without natriuretic peptides (at concentrations from 1pM to 1 μM). In these conditions, all of the peptides tested resulted in a concentration-dependent inhibition of the cyclic AMP levels, reaching about 40% inhibition in each case (Figure 30A). Uninjected oocytes showed no such inhibition by these peptides.

Identical experiments using VIP-related neuropeptides instead of natriuretic peptides were also performed. It was observed that VIP and certain related peptides were also able to dose-dependently inhibit FSK-induced cAMP elevation (Figure 30B). Among the neuropeptides tested, VIP, PHI and PACAP-27 showed a maximum 42-50% inhibition of the FSK-induced basal level, with IC50s of about 1nM. However, PACAP-38, which only poorly displaced radiolabeled ANP, did not significantly inhibit the FSK-induced cAMP accumulation.

The cloning of the PACAP-preferring PAC1 receptors in Xenopus (Hu Z, et al. (2000a) *Endocrinol*, 141: 657-665) was previously reported. This receptor in other species is primarily positively coupled to adenylyl cyclase activity. The Xenopus PAC1 mRNA was injected as described above into oocytes. Oocytes (10/sample) were preincubated within a solution of 1 mM IBMX and 2 μM FSK for 15 min at room temperature prior addition of the specified peptides. Peptides were incubated for another 15 min period, before cAMP extraction. Cyclic AMP concentrations were measured by RIA. Experiments were conducted in triplicates. Pre-treatment of injected oocytes with IBMX 1 mM and FSK 2 μM produced an overall 6-fold induction of cAMP basal levels. Subsequent addition for 15 minutes of neuropeptides PACAP-38 and PACAP-27 triggered a potent stimulation of cAMP contents, but no significant cAMP production was recorded when VIP or PHI was incubated within the oocytes (Figure 31A). No efficient activation of adenylyl cyclase was reported when PAC1 mRNA injected oocytes were stimulated with natriuretic peptides (Figure 31B), or when uninjected oocytes were incubated with natriuretic, VIP, PHI, or PACAP peptides.

PAC1 and XNPR-C receptor mRNAs were co-injected into oocytes to study the interaction of these 2 receptors on AC activity. Oocytes (10/sample) were preincubated for 15 min at room temperature, within a solution of 1 mM IBMX also containing 2μM FSK (A and B), or 50 nM PACAP (C) or 50 nM ANP (D). The specified peptides were then incubated for another 15 min period, before cAMP extraction. Cyclic AMP concentrations were measured by RIA. Experiments were conducted in triplicates. As previously described, oocytes were pre-stimulated with 2 μM FSK. This induced a 4-fold increase of the basal level in co-injected oocytes. Subsequent 15-minute stimulation with natriuretic peptides induced a dose-dependent inhibition of the FSK-induced cAMP levels (Figure 32A). VIP also produced a dose-dependent 38% inhibition of cAMP levels, comparable to that observed in oocytes expressing NPR-C alone. In contrast, PACAP-38 induced a 2-fold stimulation of cAMP levels in co-injected oocytes (Figure 32B). In addition, PACAP-27 had little effect on FSK-induced cAMP levels, producing significant inhibition only at the highest

concentration tested.

To determine if natriuretic peptides or VIP (acting primarily on NPR-C) have an effect on PACAP-38/PAC1 induction of cAMP, oocytes were coinjected with XNPR-C and PAC1 mRNAs. In control experiments, coinjected oocytes were incubated with or without 50 nM PACAP-38 for 30 min. A low concentration of FSK, 2 μM, was included in the incubation medium to keep these experiments consistent with those shown in Figure 31. Levels of cAMP were 40% higher when cells were treated with PACAP-38 than when treated with FSK alone (Figure 32C). Addition of ANP or ANP analogs for the final 15 minutes resulted in a 35 to 41% reduction of the PACAP-induced cAMP levels. Moreover, the same phenomenon was observed when VIP replaced natriuretic peptides, leading to a VIP-induced inhibition of the PACAP-induced stimulation of adenylyl-cyclase.

In coinjected oocytes, it was determined if PACAP-38, acting primarily through PAC1 receptors, could counteract the NPR-C mediated-inhibition of cAMP levels. To test this, previous experiments were repeated, but in reversed protocol with the addition of PACAP-38 in the final 15 minutes. In control experiments, oocytes were incubated for 30 min with 2 μM FSK with or without 50 nM ANP. Inclusion of ANP resulted in cAMP levels that were 38% lower than with FSK alone (Figure 32D). Addition of PACAP-38 in the final 15 min resulted in a relatively small increase in cAMP contents, with levels 2-fold higher at the highest PACAP-38 concentration tested (1 μM). In the same conditions, PACAP-27 showed an even smaller induction of cAMP levels.

Discussion

Analysis of X-NPRC receptors

This example reports the isolation and the molecular characterization of a Xenopus NPR-C receptor. Sequence analysis revealed a Xenopus NPR-C receptor encoding a protein of 523 amino acids. The open reading frame contained characteristic features of non-GC

natriuretic peptide receptors. This includes a transmembrane spanning domain located in position 447-467, a short intracellular tail of 35 amino acids, 3 putative sites (NXS/T) for N-glycosylation, as well as 6 cysteine residues that may be involved within intra- and/or inter-chain interactions. Such features suggested the model depicted in Figure 27. In addition, high identities in the protein sequences have been found in positions 98-117. This conforms to the proposed natriuretic receptor signature (GPXCXYXXAXVXRXXXHW) (Takashima et al, (1995) *Eur J Biochem*, 227: 673-680).

Computer-assisted multialignment of the previously published sequences of NPR-C from mammalians and fish with the *Xenopus* NPR-C receptor revealed that the sequence was closer in homology to mammals than eel. Interestingly, among all the known mammalian sequences, the highest homology observed was with the human sequence.

The XNPR-C receptor was also compared to all *Xenopus* sequences available in the database. Two Xenopus sequences had significant homology (Figure 26). These encode two guanylate-cyclase containing proteins, named XGC-1 and XGC-2 (Genbank access number, AB025111.1 and AB025111.2, respectively). Like XNPR-C, XGC sequences had an extracellular domain, a putative transmembrane domain, but included a very long intracellular tail that contained the guanylate cyclase activity. Moreover, widely spread homologies with the XNPR-C sequence were observed in the extracellular region. Among the numerous similarities, the perfect alignment and conservation of all the 6 cysteine residues was noteworthy. Moreover, the NP receptor signature domain (GPXCXYXXAXVXRXXXHW) was conserved within these 3 *Xenopus* sequences.

Binding activity and ligand specificity

Sequence analysis revealed that the histidine and tryptophan residues in position 145-146 were important for the binding activity (Iwashina M, et al. (1994) *J Biochem*, 115: 563-567) and were conserved in the present *Xenopus* sequence. In addition, an analysis of the

binding characteristics of the expressed mRNA was performed. Saturation experiments showed that injected oocytes expressed a very large number of NPR-C receptors (Bmax about 57 millions receptors/oocyte) with a subnanomolar affinity for ANP (K_D of 97 pM). These data are in agreement with the pharmacological parameters previously published for PAC1 (Hu Z, et al. (2000) *Endocrinol*, 141: 657-665).

Specificity of the binding sites for the different natriuretic peptides was analyzed using classical binding displacement experiments. This XNPR-C receptor showed a relative lack of specificity for the different natriuretic peptides and analogs. As indicated in Table III, all frog and rat analogs tested had an IC_{50} between 0.09 and 2.1 nM. This was a common feature of all cloned NPR-C receptors. Moreover, this XNPR-C receptor possessed a high affinity for the $desANP_{4-24}$, and binding was insensitive to HS142.1. These latter characteristics have been used previously to distinguish mammalian NPR-C from NPR-A and NPR-B.

In this example, the ability of VIP-related neuropeptides to displace the radiolabeled ANP binding on oocytes expressing cloned XNPRC was studied. The data clearly demonstrate that the expressed ^{125}I-ANP binding sites displayed significant affinity for these neuropeptides. VIP and PHI showed IC_{50} of 0.19 and 0.38 nM, respectively, which was approximately equivalent to their relative affinity for the cloned *Rana* VPACs (Vaudry et al, 2000, *Pharm. Rev.*, 52, 269-324). XNPR-C also had a significant affinity for PACAP-27, but not for PACAP-38. Overall, the binding studies indicte that XNPR-C is polyvalent for natriuretic and VIP-related peptides, and discriminated between the two common forms of PACAP.

Clearance vs. signaling function of XNPR-C

Type-C natriuretic peptide receptors have been long considered to be clearance receptors for circulating natriuretic peptides. The clearance function appears to require dimerization of NPR-C monomers and internalization of the ligand/receptor complex. Modification of

the human receptor sequences affecting the residues Cysteine[473] or the tyrosine[508] triggered a 50% inhibition of the receptor internalization (Cohen D, et al. (1996). *J Biol Chem*, 271: 9863-9869). The present Xenopus NPR-C receptor showed a perfect identity of sequence in these crucial positions. More recently, targeted NPR-C gene disruption (Matsukawa N, et al. (1999) *Proc Natl Acad Sci U S A*, 96: 7403-7408) or spontaneous mutation/deletion in the NPR-C locus (Jaubert et al, 1999, *Proc. Natl. Acad. Sci.* 96, 10278-10283) was associated with a severe phenotype in mice. These mice exhibited a pronounced overgrowth of the skeleton. A similar phenotype was observed in transgenic mice that overexpressed CNP (Suda M, et al. (1998). *Proc Natl Acad Sci U S A.* 95: 2337-42). This bone overgrowth was thought to be mediated by CNP through NPR-B receptors (Hagiwara H, et al. (1994) *J Biol Chem*, 269: 10729-10733; Hagiwara H, et al. (1996) *Am J Physiol*, 270, C1311-C1318; Yasoda A, et al. (1998) *J. Biol Chem*, 273: 11695-11700). Taken together, these data indicated that NPRC is involved in the regulation of skeleton growth, through its clearance function of CNP. Because XNPR-C receptors were found to bind VIP and related peptides with high affinity in the present study, NPR-C might also function as a clearance receptor for certain VIP-related neuropeptides.

In addition to the clearance function, recent studies have suggested that NPR-C might also be coupled to a signal transduction pathway. Studies performed by Anand-Srivastava (Anand-Srivastava MB and Trachte GT (1994) *Pharmacol Rev*, 45: 455-497, Anand-Srivastava MB, et al. (1996) *J Biol Chem*, 271: 19324-19329) and others (Murthy KS, and Makhlouf GM (1999) *J Biol Chem.* 274:17587-17592) proposed that NPR-C activation results in an inhibition of adenylyl cyclase activity and/or increased intracellular calcium through a pertussis toxin-sensitive Gi. Moreover, a recent study identified the intracellular sequence of NPR-C that is involved in Gi coupling (Anand-Srivastava MB, et al. (1996) *J Biol Chem*, 271: 19324-19329). This sequence is conserved in the cloned XNPR-C receptor reinforcing the need for testing the negative coupling of the XNPR-C receptors to adenylyl cyclase. This hypothesis was assessed by measuring cAMP contents in NPRC-overexpressing oocytes after stimulation with various

natriuretic peptides. The data confirmed this coupling of NPR-C receptors to adenylyl cyclase, since ANP and other analogs reduced by about 50% the level of intracellular cAMP induced by forskolin. This inhibition was dose-dependent, and the efficiency of natriuretic peptides to inhibit the FSK-induced cAMP formation correlated with their relative affinity for this receptor. In addition, VIP, PHI and PACAP-27, which displaced significant affinity for XNPR-C, significantly reduced the FSK-induced intracellular levels of cAMP, whereas PACAP-38, which showed a very low affinity for XNPR-C, was unable to do this.

The isolation and characterization of the *Xenopus* PACAP-preferring (PAC1) receptor (Hu Z, et al. (2000a) *Endocrinol*, 141: 657-665) was recently reported. Among its characteristics, it displayed particularly high affinity for PACAP-38, a lower affinity for PACAP-27, and no significant affinity for VIP. Like PAC1 receptors cloned in various other species, it showed strong coupling to adenylyl cyclase (Vaudry et al, 2000, *Pharm. Rev.*, 52, 269-324).

This example suggests that peptides acting on NPR-C regulator might regulate PACAP/PAC1 activity in Xenopus. Injection of NPR-C and PAC1 mRNAs alone and in combination were used to test this hypothesis. First, as a control of the dual receptor system, the ability of natriuretic peptides and neuropeptides to modulate the FSK-induced elevation of cAMP was tested. Natriuretic peptides, as expected, reduced the cAMP contents, through NPR-C receptors but did not interact with PAC1 receptors. Conversely, PACAP-38 significantly enhanced the FSK action on cAMP production. Between these extreme cases, PACAP-27, which interacted with each of the receptors, displayed an intermediate behavior.

To further investigate these possible antagonistic interactions of NPRC and PAC1 on the activation of adenylyl cyclase, the FSK pre-incubation was replaced by either 50 nM PACAP-38 or ANP. Antagonistic effects on cAMP production were studied by treatment with natriuretic peptides and neuropeptides. First, PACAP-38–induced stimulation of

cAMP contents was abolished and reverted by either natriuretic peptides or VIP analogs through their interaction with NPR-C receptors coupled negatively with AC. Second, ANP-induced-inhibition of cAMP basal levels was counterbalanced by PACAP stimulations. However, PACAP-38 and PACAP-27 stimulatory effects were strongly reduced by co-stimulation of NPR-C receptors. These results clearly demonstrate that NPR-C receptors may act as a negative regulator of PACAP actions.

We Claim:

1. A method for regulating neuronal cell growth, proliferation or survival comprising contacting cells expressing a natriuretic peptide receptor with a compound in an amount effective to regulate neuronal cell growth, proliferation or survival.

2. A method of treating a disease associated with aberrant neuronal cell growth or proliferation comprising contacting cells expressing a natriuretic peptide receptor with a compound in an amount effective to inhibit said neuronal cell growth or proliferation.

3. A method of treating neuronal injury associated with neuronal degeneration comprising contacting cells expressing a natriuretic peptide receptor with a compound in an amount effective to enhance neuronal cell survival associated with neuronal injury.

4. The method of claim 1, 2 or 3, wherein said natriuretic peptide receptor is selected from the group consisting of Type A, B and C receptors.

5. The method of claim 1, 2 or 3, wherein said compound is a natriuretic peptide or PHI-related peptides.

6. The method of claim 5, wherein said natriuretic peptide is selected from the group consisting of atrial natriuretic peptide, brain natriuretic peptide, and type-C natriuretic peptide.

7. The method of claim 5, wherein said PHI-related peptide is selected from the group consisting of PHI, VIP and PACAP.

8. The method of claim 1, 2 or 3, wherein said compound is an antisense oligonucleotide molecule complementary to the nucleotide sequence of the natriuretic peptide receptor.

9. The method of claim 1, 2 or 3, wherein said compound is an antisense oligonucleotide molecule complementary to the nucleotide sequence of natriuretic peptides.

10. The method of claim 9, wherein said natriuretic peptide is selected from the group consisting of atrial natriuretic peptide, brain natriuretic peptide, and type-C natriuretic peptide.

11. The method of claim 2, wherein the disease is selected from the group consisting of neurodegenerative diseases, neuronal tumors, Huntington's disease, Alzheimer's disease, epilepsy, lathyrism, amyotrophic laterial sclerosis, and Parkinsonian dementia.

12. A pharmaceutical composition comprising a compound selected from the group consisting of an antisense oligonucleotide molecule complementary to the nucleotide sequence of natriuretic peptide receptor, an antisense oligonucleotide molecule complementary to the nucleotide sequence of natriuretic peptides, a PHI-related peptide and a natriuretic peptide.

13. An isolated nucleic acid molecule comprising the nucleotide sequence encoding a polypeptide as shown in Figure 24.

14. The nucleic acid molecule of claim 13 having Genbank Accession No. AF231035.

15. An isolated protein comprising the amino acid sequence as shown in Figure 24.

16. A method of detecting a tumor cell expressing a natriuretic peptide receptor comprising contacting cells expressing a natriuretic peptide receptor with a ligand for the receptor, where said ligand is labeled for detection.

5

17. The method of claim 16, wherein said ligand is an antibody that binds to said receptor.

10

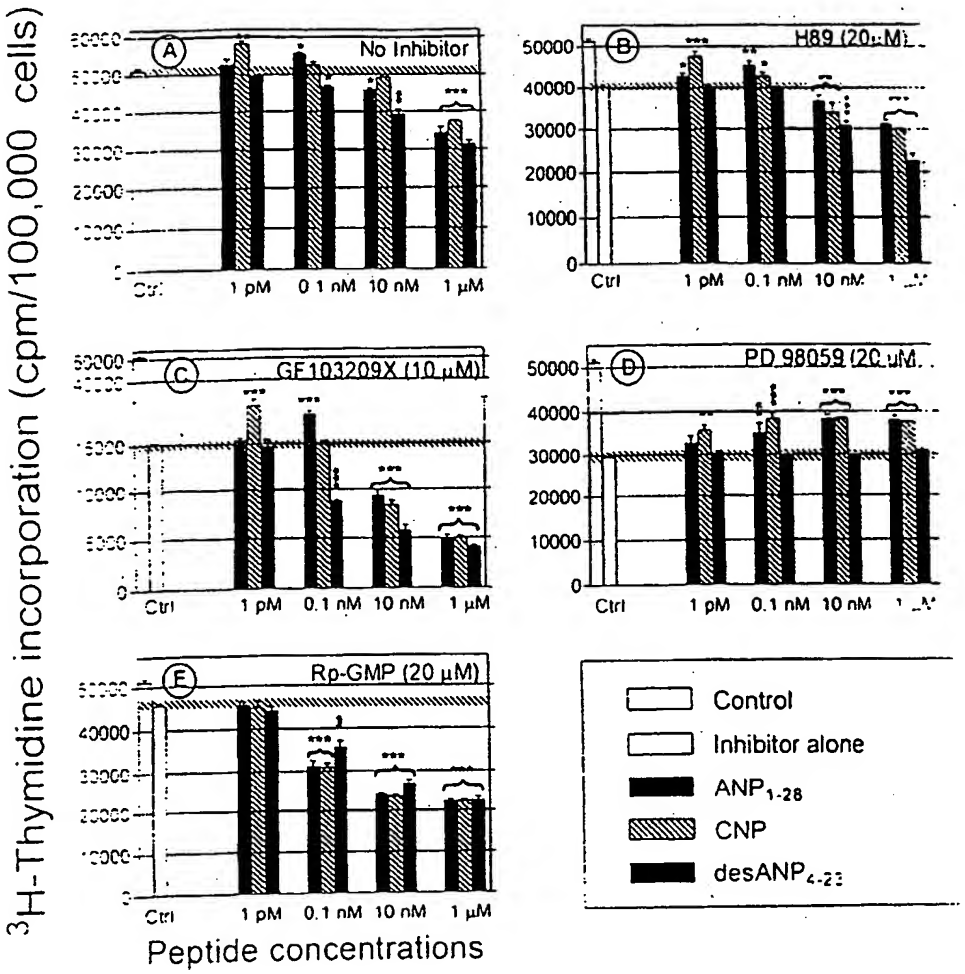

^{3}H-Thymidine incorporation (cpm/100,000 cells)
A
No Inhibitor
B
H89 (20 µM)
C
GF103209X (10 µM)
D
PD 98059 (20 µM)
E
Rp-GMP (20 µM)
Ctrl
1 pM
0.1 nM
10 nM
1 µM
Peptide concentrations
Control
Inhibitor alone
ANP1-28
CNP
desANP4-23

Figure 1

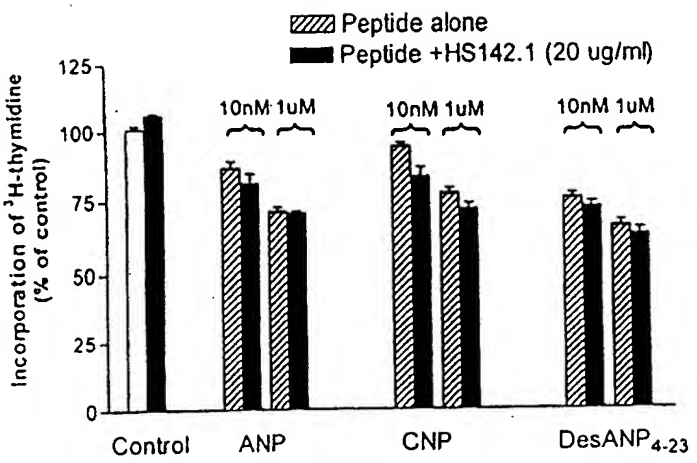

Peptide alone
Peptide +HS142.1 (20 ug/ml)
Incorporation of ³H-thymidine (% of control)
125
100
75
50
25
0
10nM 1uM
10nM 1uM
10nM 1uM
Control
ANP
CNP
DesANP4-23

Figure 2

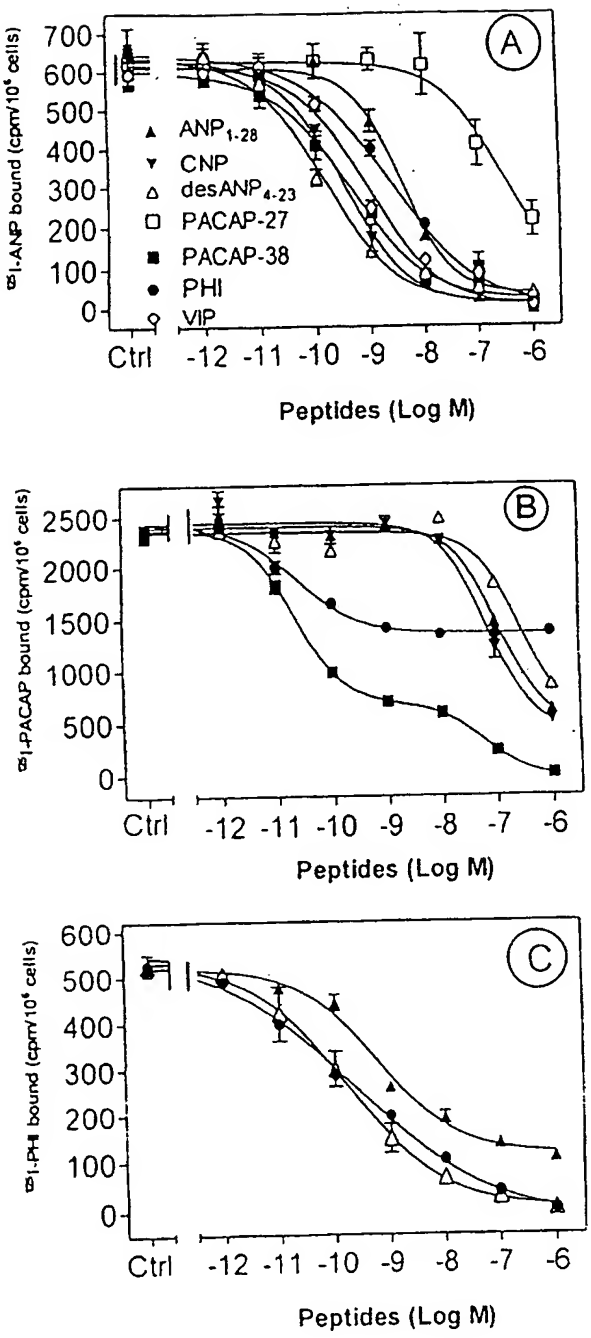

A
B
C
ANP1-28
CNP
desANP4-23
PACAP-27
PACAP-38
PHI
VIP
Ctrl
-12 -11 -10 -9 -8 -7 -6
Peptides (Log M)

Figure 3

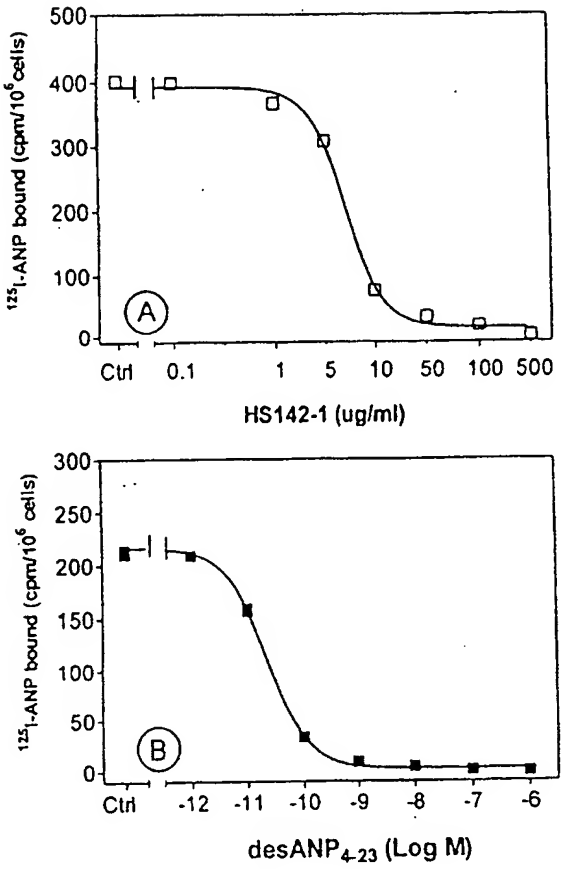

500
400
300
200
100
0
^{125}I-ANP bound (cpm/10^6 cells)
A
Ctrl
0.1
1
5
10
50
100
500
HS142-1 (ug/ml)
300
250
200
150
100
50
0
^{125}I-ANP bound (cpm/10^6 cells)
B
Ctrl
-12
-11
-10
-9
-8
-7
-6
desANP$_{4-23}$ (Log M)

Figure 4

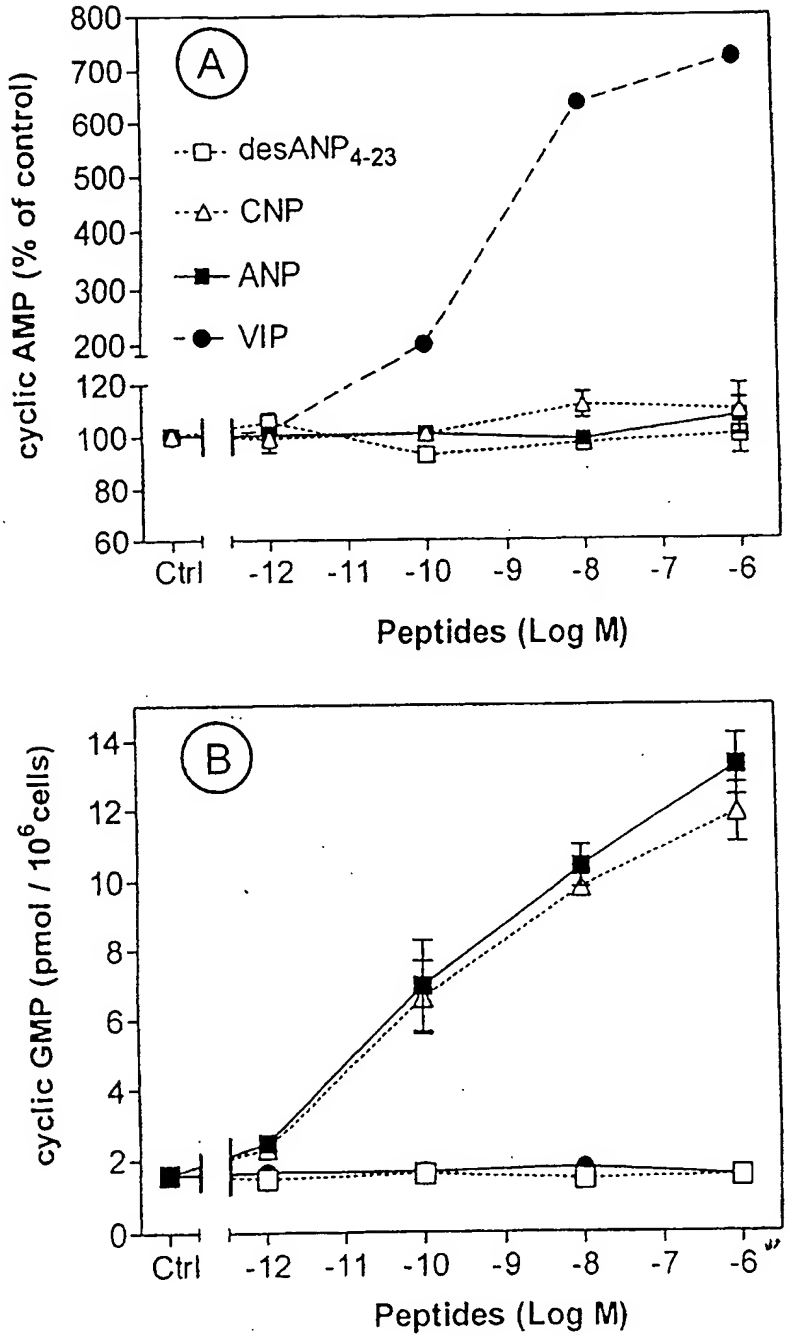

A
desANP4-23
CNP
ANP
VIP
cyclic AMP (% of control)
800
700
600
500
400
300
200
120
100
80
60
Ctrl
-12
-11
-10
-9
-8
-7
-6
Peptides (Log M)
B
cyclic GMP (pmol / 10^6 cells)
14
12
10
8
6
4
2
0
Ctrl
-12
-11
-10
-9
-8
-7
-6
Peptides (Log M)

Figure 5

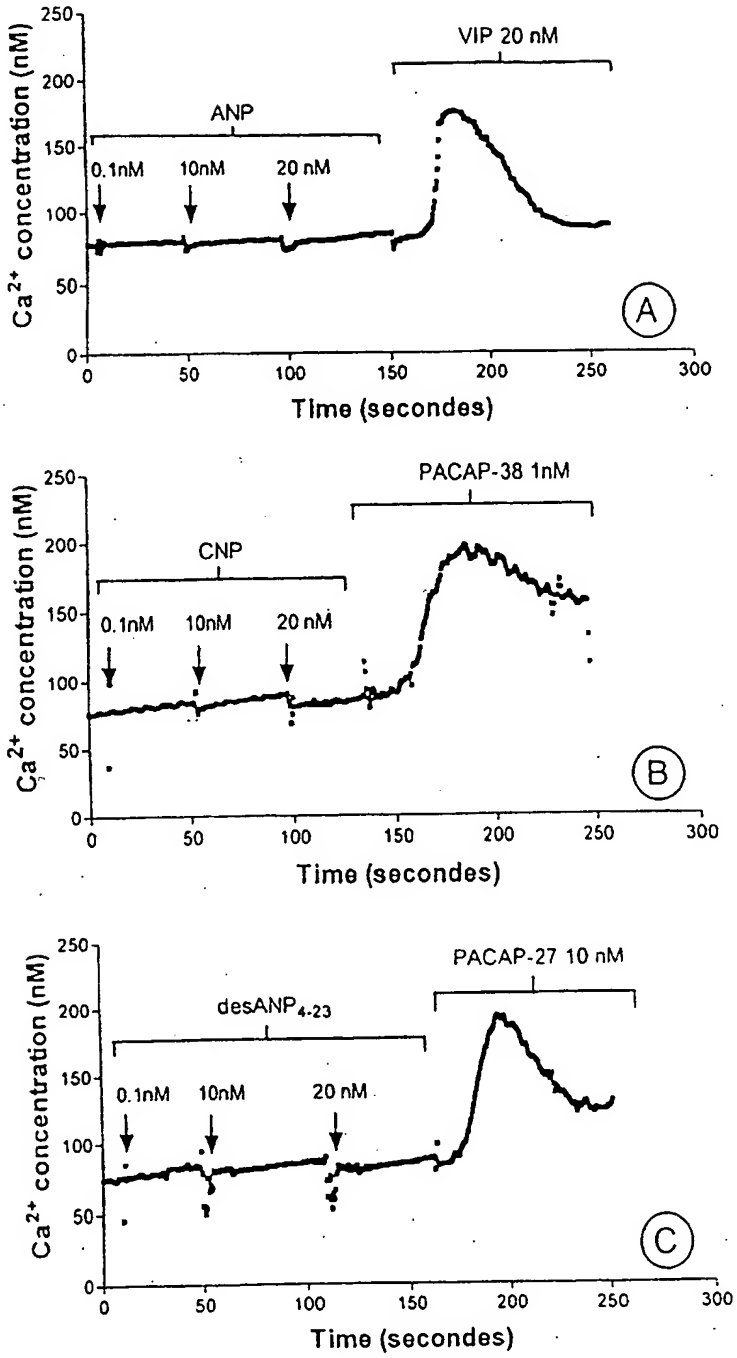

VIP 20 nM
ANP
0.1nM
10nM
20 nM
Ca^{2+} concentration (nM)
Time (secondes)
A
PACAP-38 1nM
CNP
0.1nM
10nM
20 nM
Ca^{2+} concentration (nM)
Time (secondes)
B
PACAP-27 10 nM
desANP4-23
0.1nM
10nM
20 nM
Ca^{2+} concentration (nM)
Time (secondes)
C

Figure 6

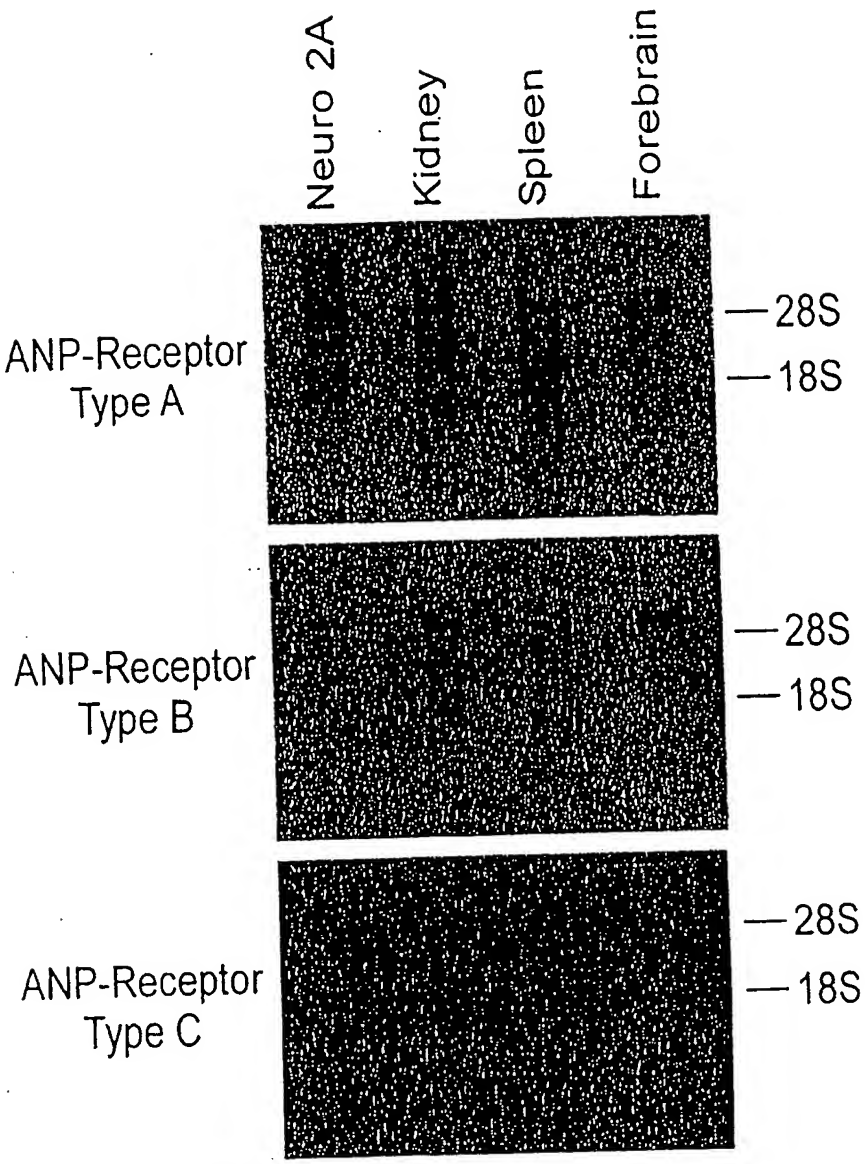
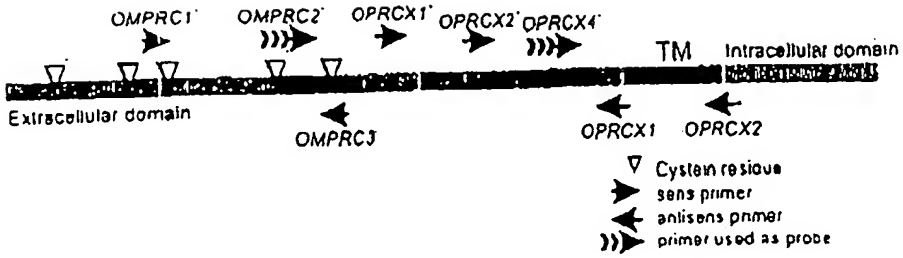

Neuro 2A
Kidney
Spleen
Forebrain
ANP-Receptor Type A
28S
18S
ANP-Receptor Type B
28S
18S
ANP-Receptor Type C
28S
18S

FIGURE 7

A


OMPRC1
OMPRC2
OPRCX1
OPRCX2
OPRCX4
TM
Intracellular domain
Extracellular domain
OMPRC3
OPRCX1
OPRCX2
Cystein residue
sens primer
antisens primer
primer used as probe

B

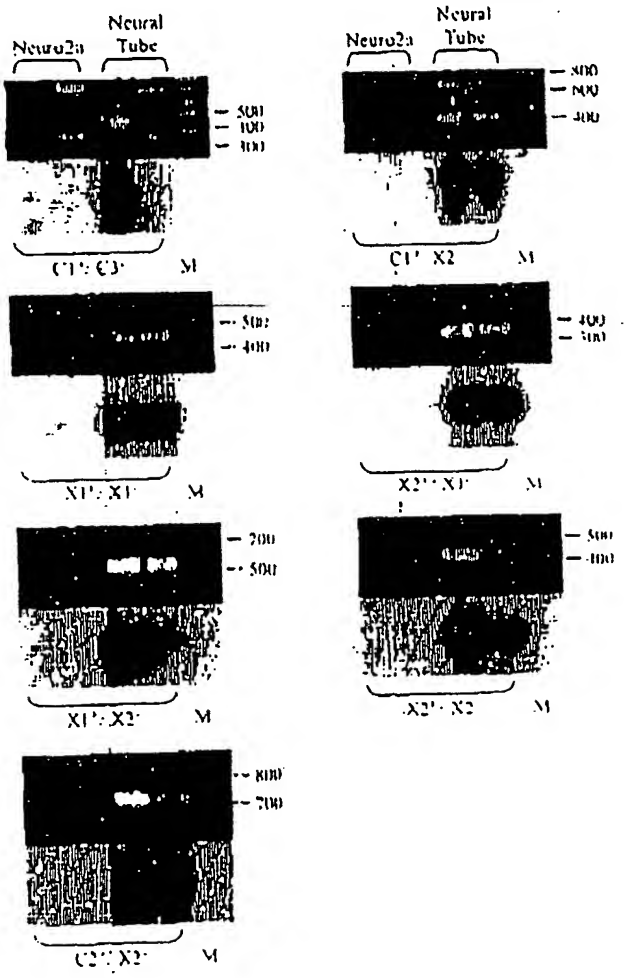

Neuro2a
Neural Tube
M

FIGURE 8

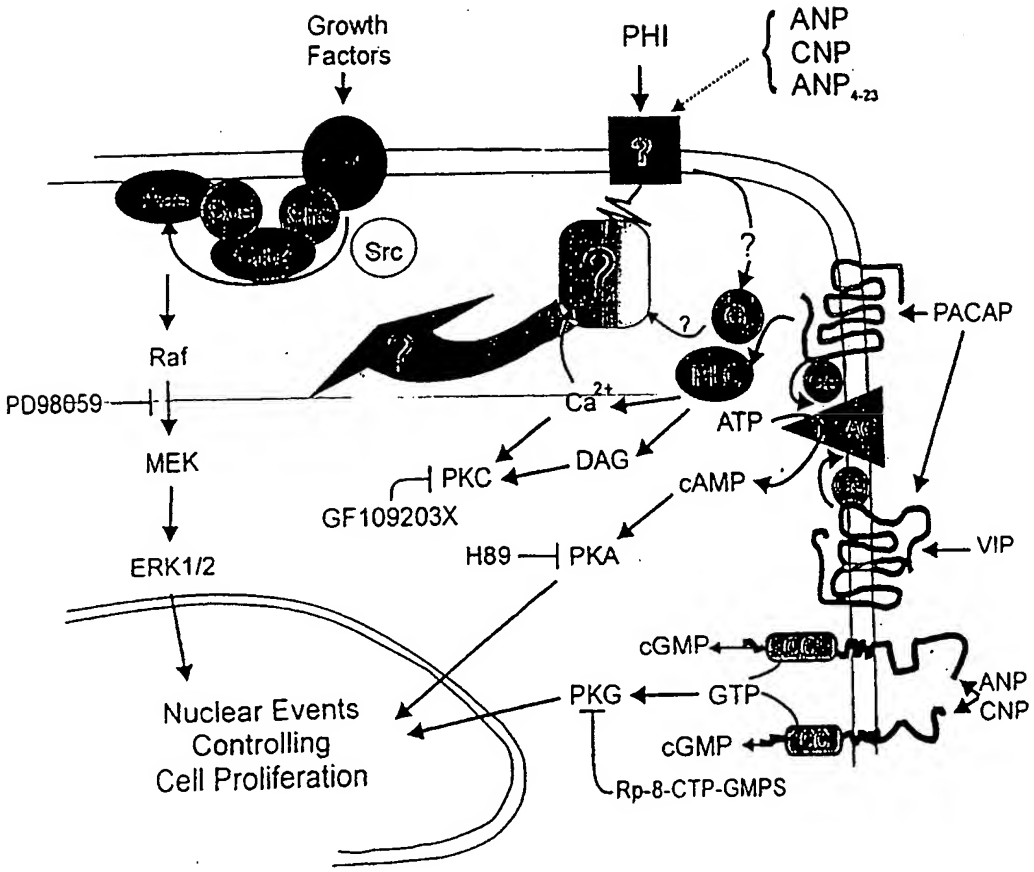

Growth Factors
PHI
ANP
CNP
ANP4-23
Src
Raf
PD98059
MEK
ERK1/2
Ca2+
ATP
DAG
PKC
GF109203X
cAMP
H89
PKA
PACAP
VIP
cGMP
GTP
PKG
Rp-8-CTP-GMPS
ANP
CNP
Nuclear Events Controlling Cell Proliferation

FIGURE 9

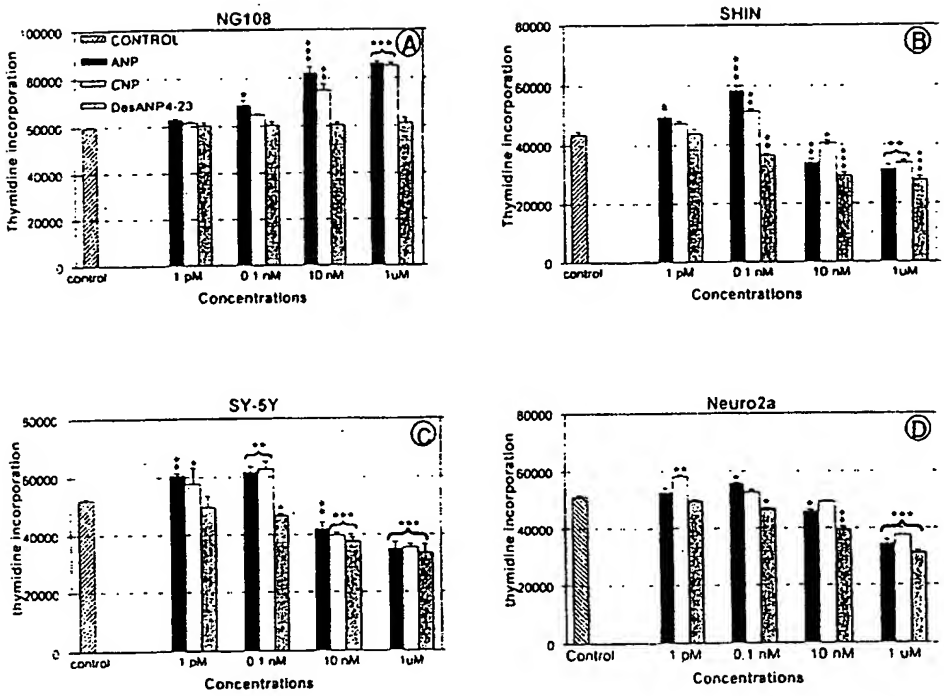

NG108
CONTROL
ANP
CNP
DesANP4-23
Thymidine incorporation
Concentrations
control
1 pM
0.1 nM
10 nM
1uM
A
SHIN
B
SY-5Y
C
Neuro2a
D
Control
1 uM

Figure 10

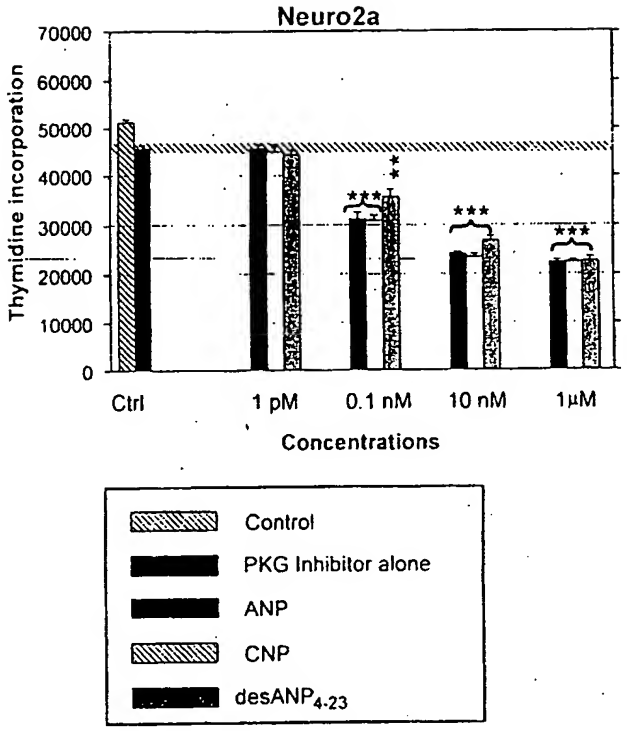

Neuro2a
Thymidine incorporation
70000
60000
50000
40000
30000
20000
10000
0
Ctrl
1 pM
0.1 nM
10 nM
1µM
Concentrations
***
**
***
***
Control
PKG Inhibitor alone
ANP
CNP
desANP4-23

Figure 11

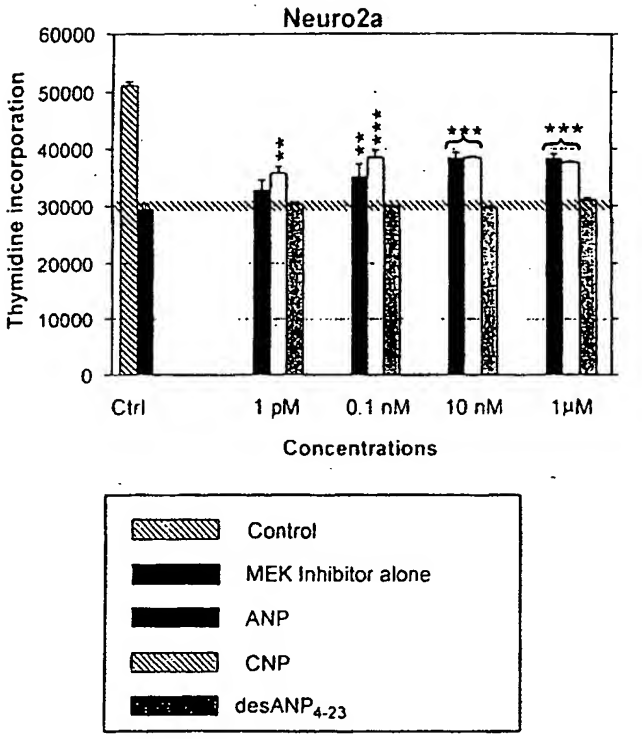

Neuro2a
Thymidine incorporation
60000
50000
40000
30000
20000
10000
0
Ctrl
1 pM
0.1 nM
10 nM
1μM
Concentrations
Control
MEK Inhibitor alone
ANP
CNP
desANP4-23

Figure 12

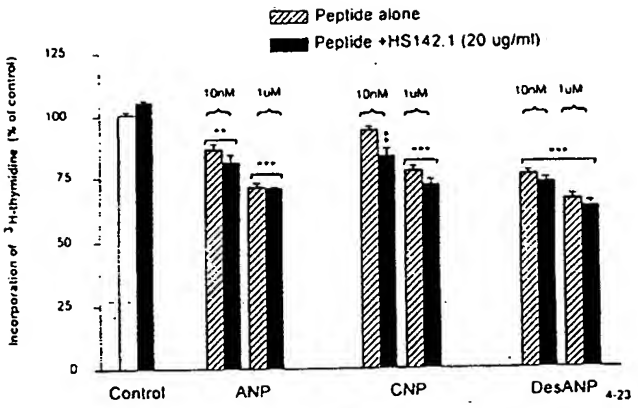

Peptide alone
Peplide +HS142.1 (20 ug/ml)
Incorporation of ^{3}H-thymidine (% of control)
125
100
75
50
25
0
10nM
1uM
Control
ANP
CNP
DesANP 4-23

Figure 13

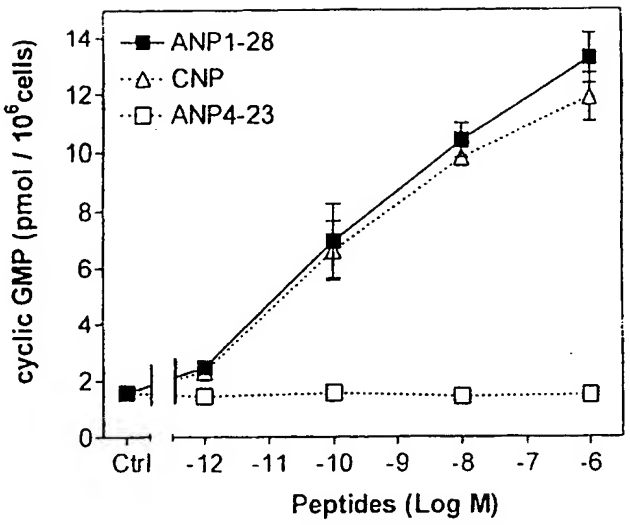

ANP1-28
CNP
ANP4-23
cyclic GMP (pmol / 10^6 cells)
0
2
4
6
8
10
12
14
Ctrl
-12
-11
-10
-9
-8
-7
-6
Peptides (Log M)

Figure 14

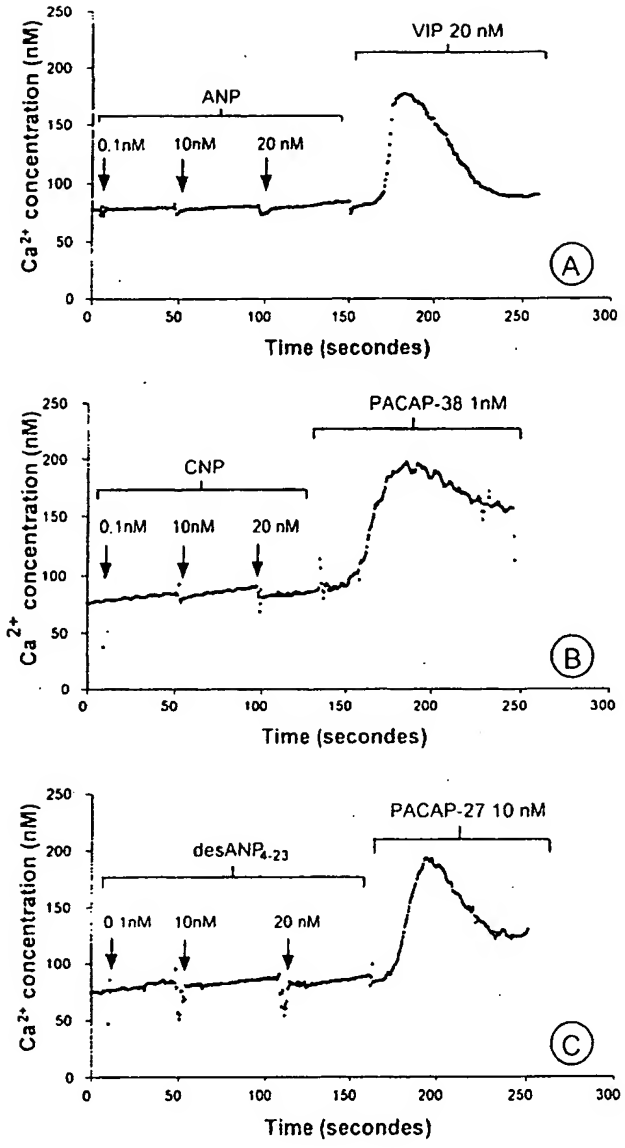

VIP 20 nM
ANP
0.1nM
10nM
20 nM
Ca2+ concentration (nM)
Time (secondes)
A
PACAP-38 1nM
CNP
0.1nM
10nM
20 nM
Ca2+ concentration (nM)
Time (secondes)
B
PACAP-27 10 nM
desANP4-23
0 1nM
10nM
20 nM
Ca2+ concentration (nM)
Time (secondes)
C

Figure 15

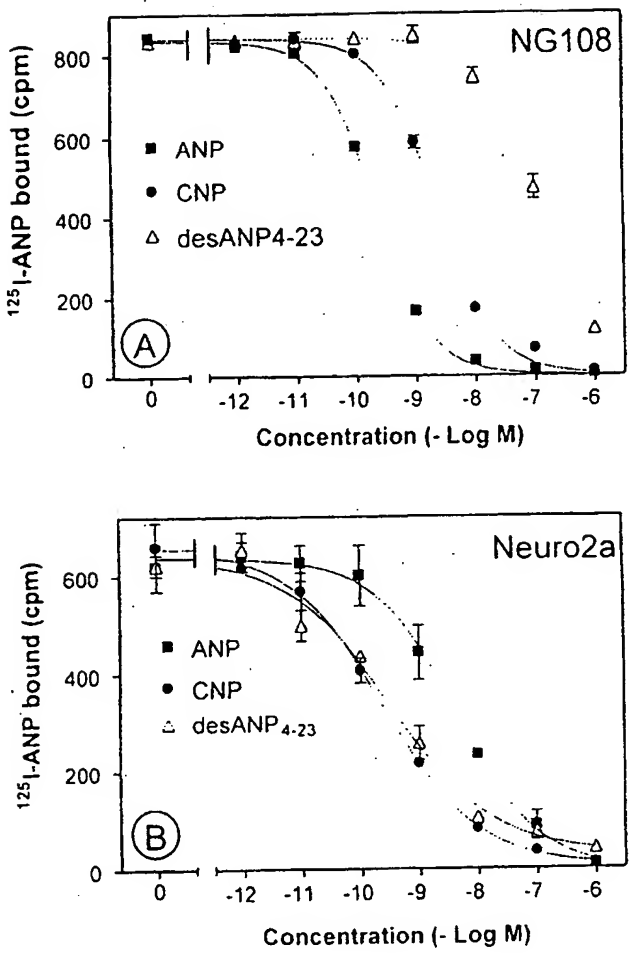

NG108
ANP
CNP
desANP4-23
A
^{125}I-ANP bound (cpm)
800
600
400
200
0
0
-12
-11
-10
-9
-8
-7
-6
Concentration (- Log M)
Neuro2a
ANP
CNP
desANP$_{4-23}$
B
^{125}I-ANP bound (cpm)
600
400
200
0
0
-12
-11
-10
-9
-8
-7
-6
Concentration (- Log M)

Figure 16

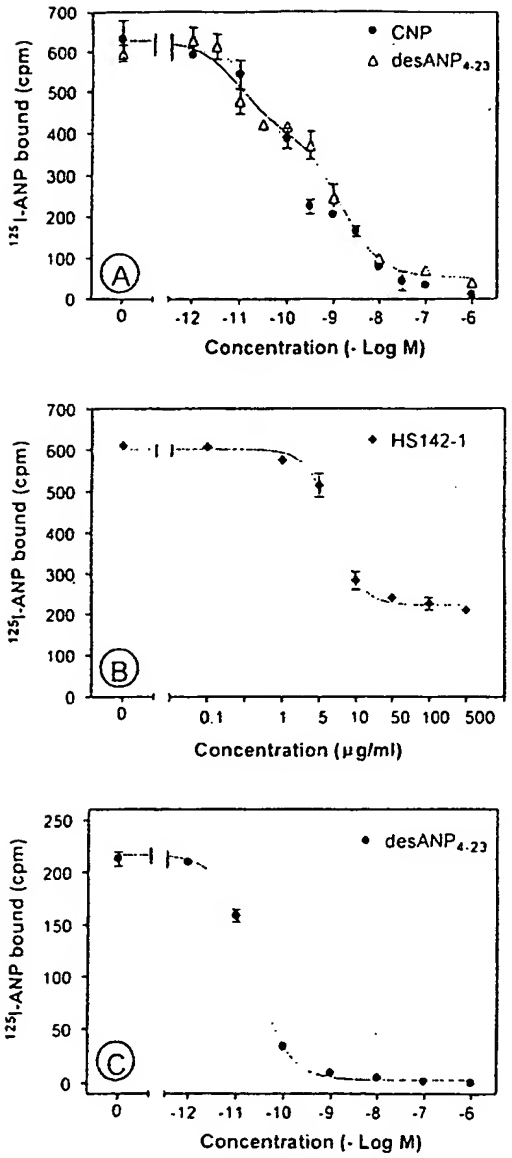

700
600
500
400
300
200
100
0
^{125}I-ANP bound (cpm)
CNP
desANP$_{4-23}$
A
0
-12
-11
-10
-9
-8
-7
-6
Concentration (- Log M)
HS142-1
B
0
0.1
1
5
10
50
100
500
Concentration (µg/ml)
250
200
150
100
50
0
desANP$_{4-23}$
C
Concentration (- Log M)

Figure 17

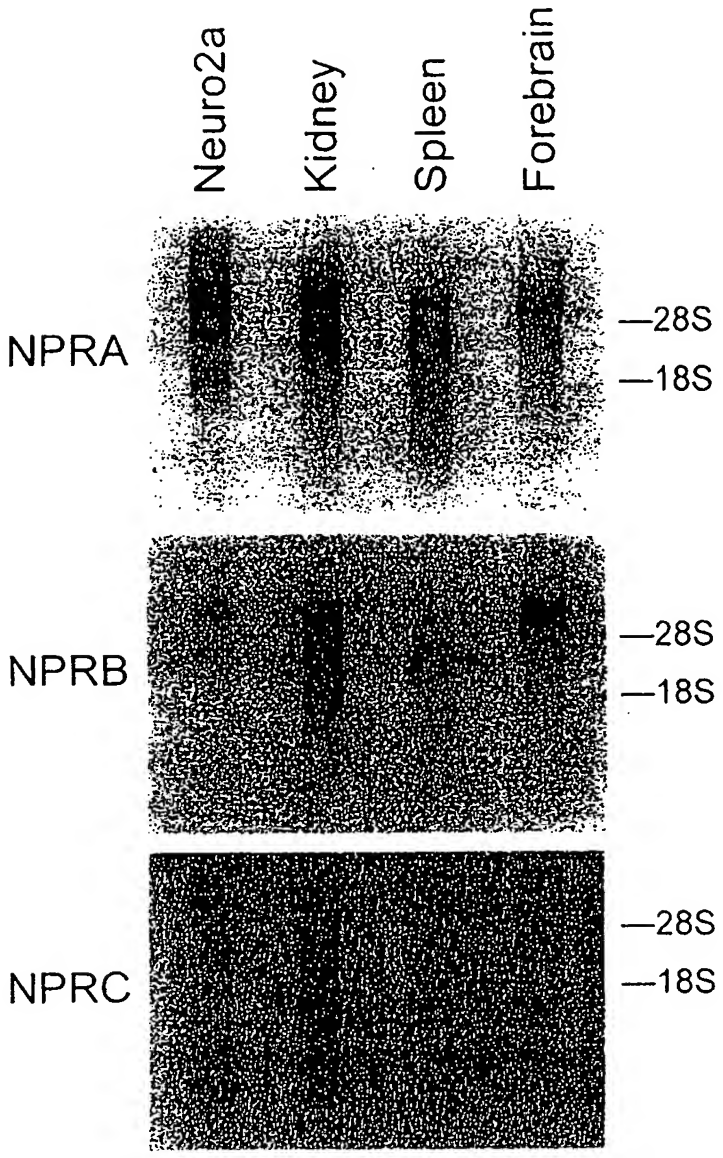

Neuro2a
Kidney
Spleen
Forebrain
NPRA
—28S
—18S
NPRB
—28S
—18S
NPRC
—28S
—18S

Figure 18

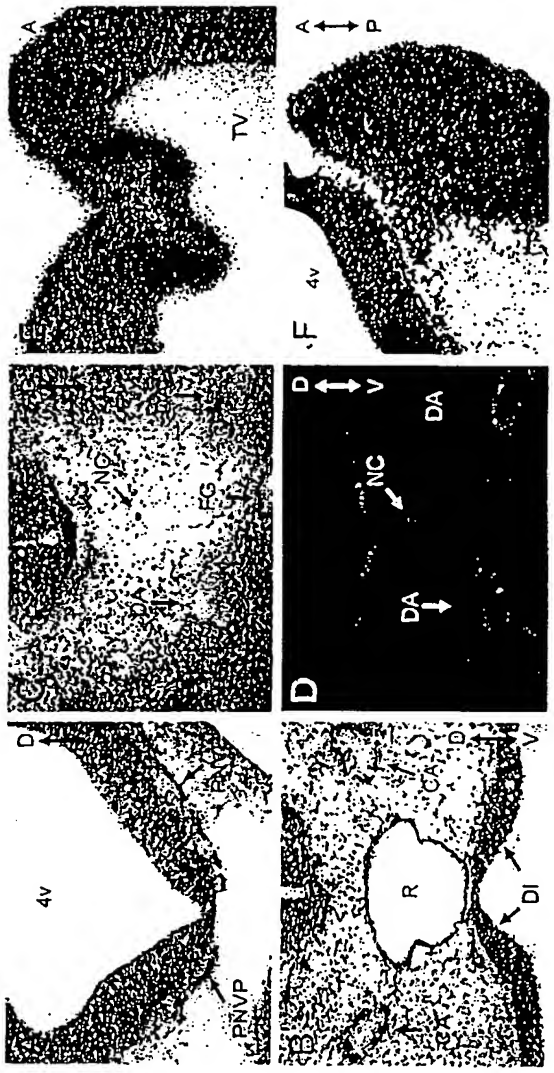


Figure 19

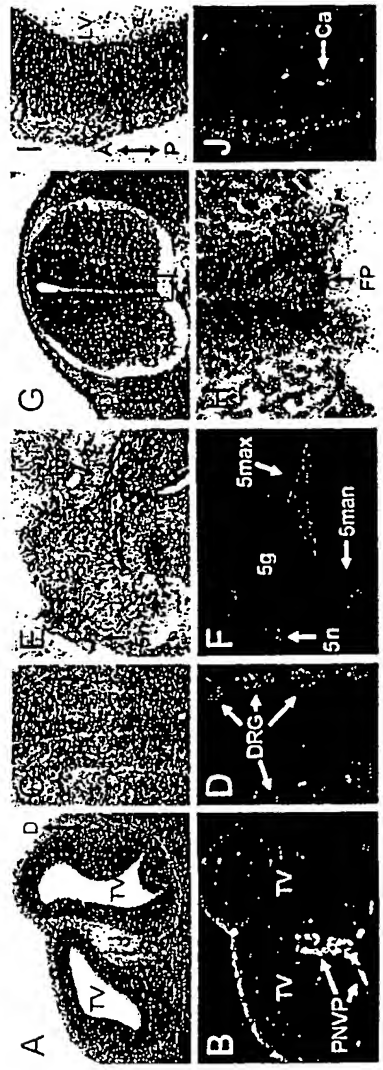


Figure 20

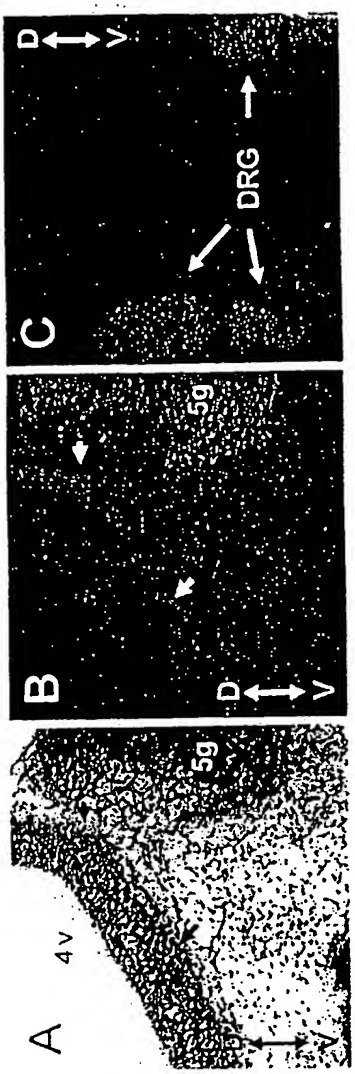


Figure 21

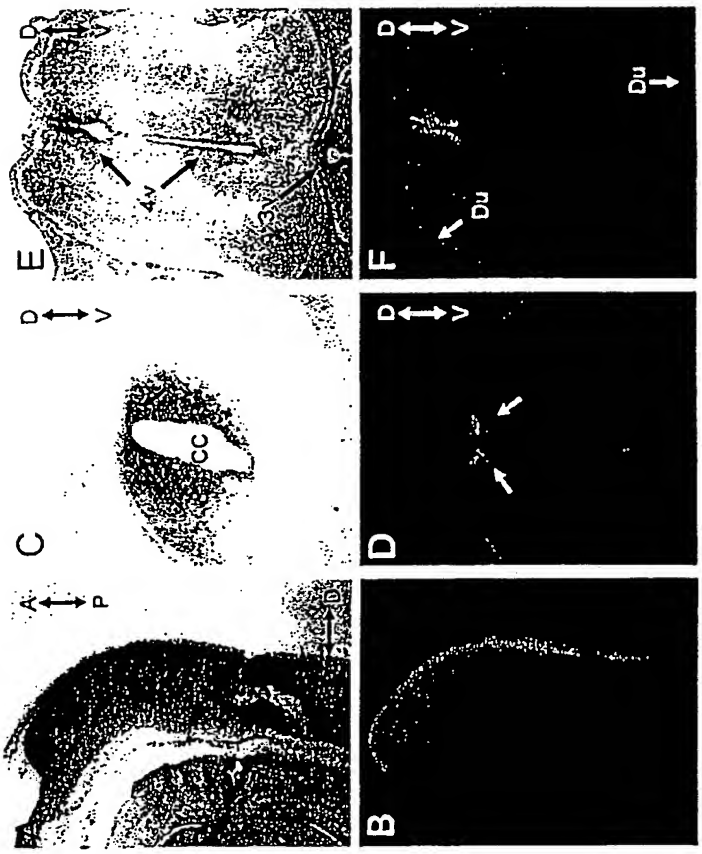


Figure 22

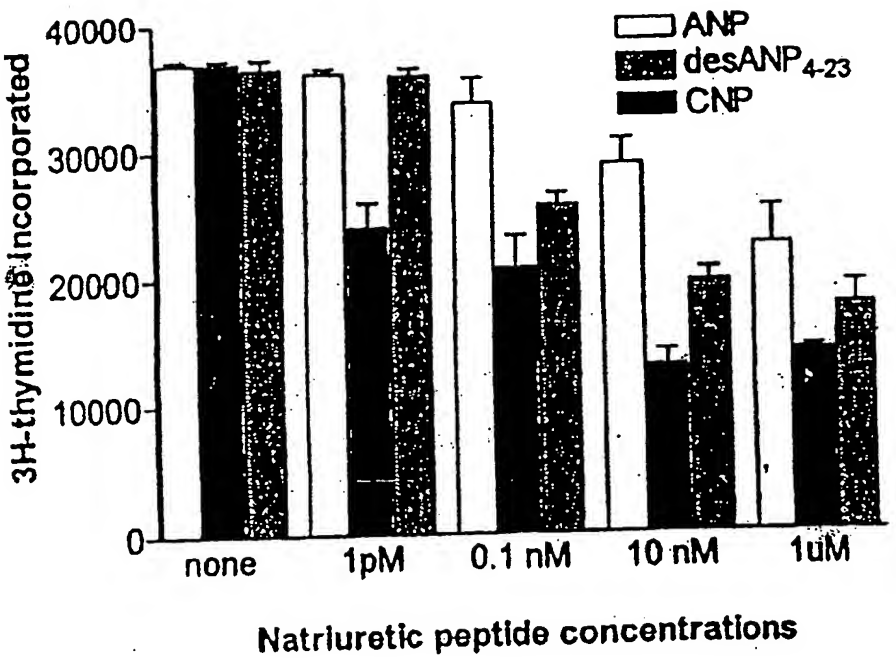

ANP
desANP4-23
CNP
40000
30000
20000
10000
0
3H-thymidine Incorporated
none
1pM
0.1 nM
10 nM
1uM
Natriuretic peptide concentrations

Figure 23

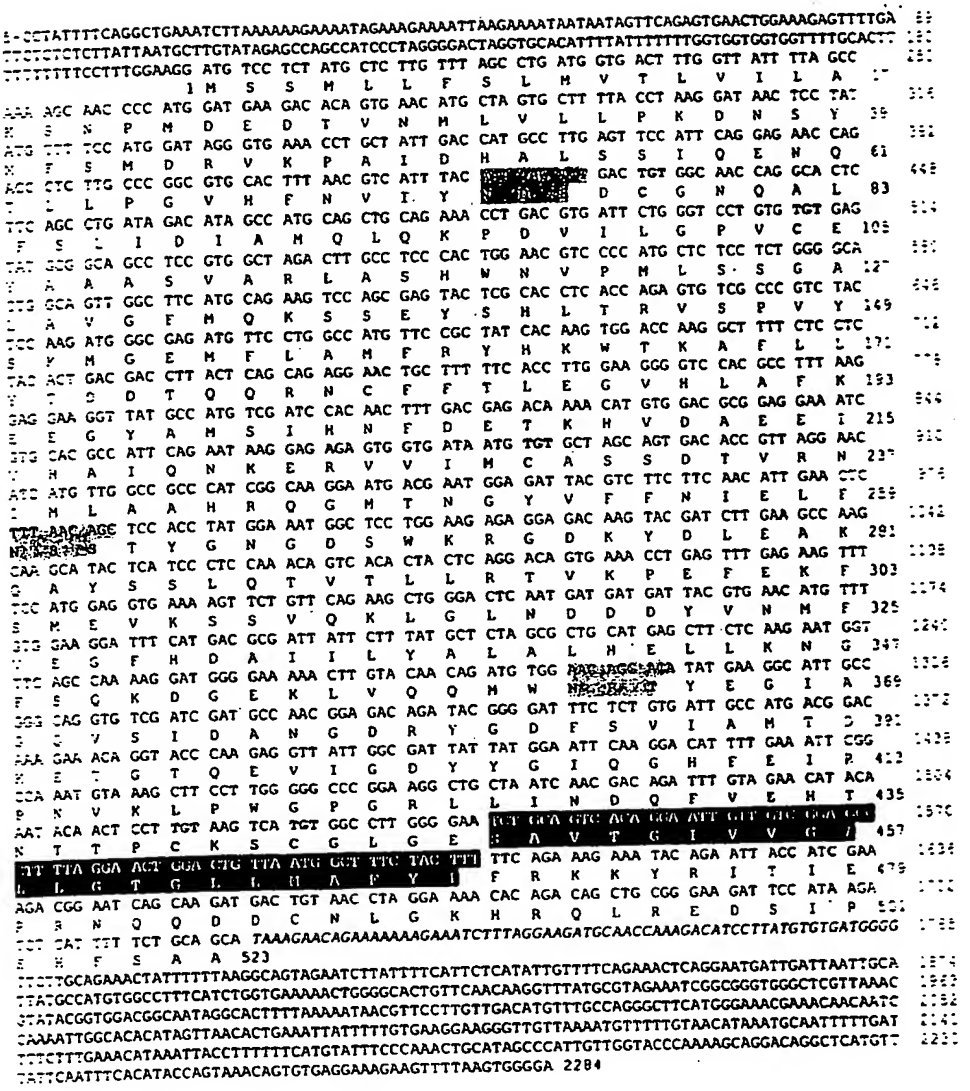


Figure 24

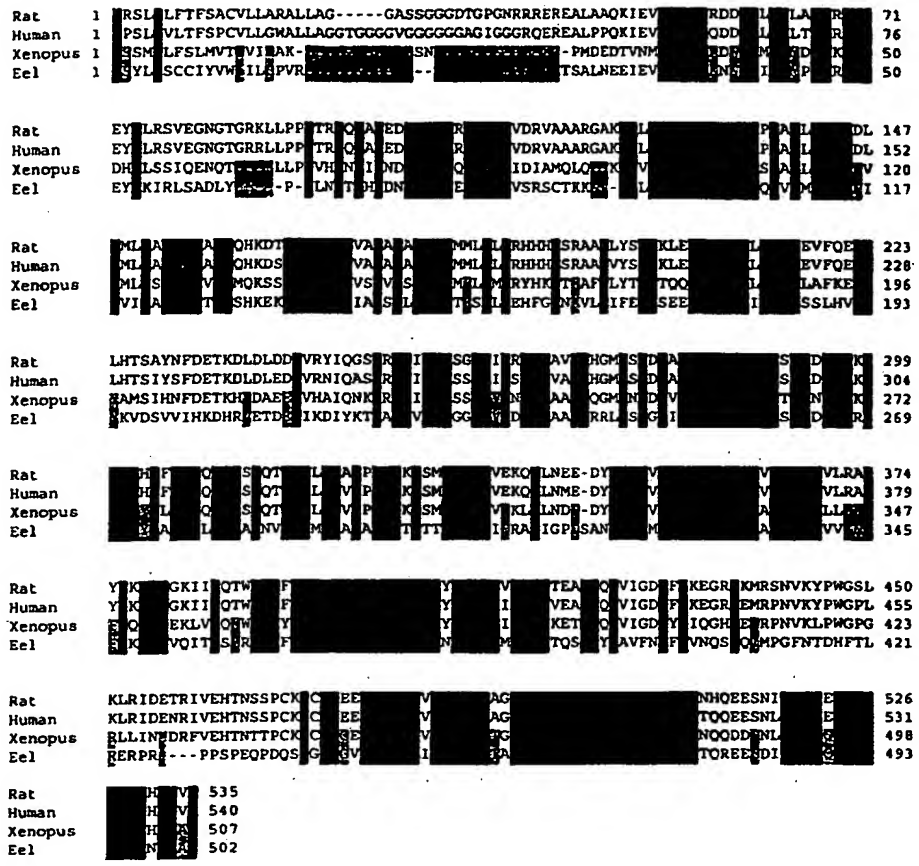


FIGURE 25

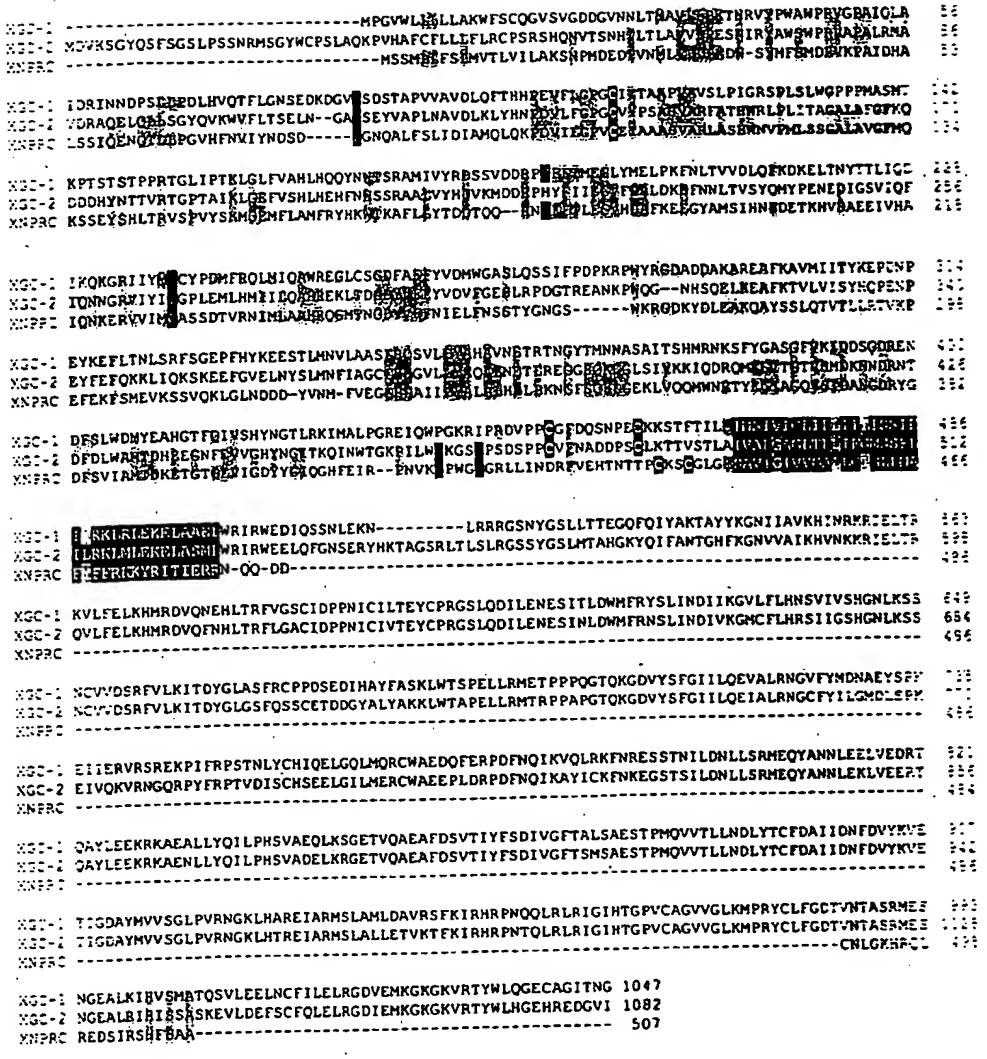


Figure 26

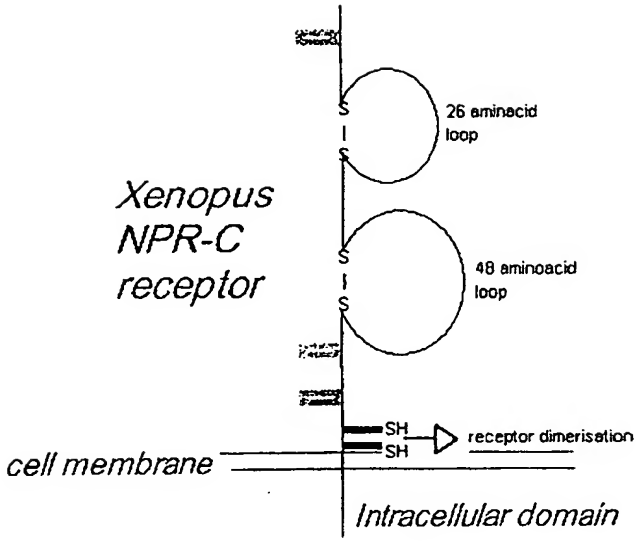

26 aminacid
loop
Xenopus
NPR-C
receptor
48 aminoacid
loop
SH
SH
receptor dimerisation
cell membrane
Intracellular domain

Figure 27

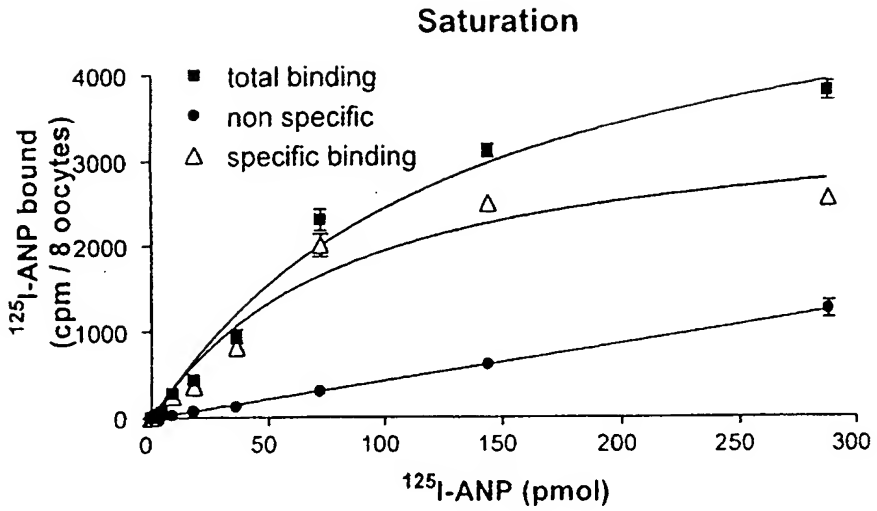

Saturation
total binding
non specific
specific binding
^{125}I-ANP bound (cpm / 8 oocytes)
4000
3000
2000
1000
0
0
50
100
150
200
250
300
^{125}I-ANP (pmol)

Figure 28

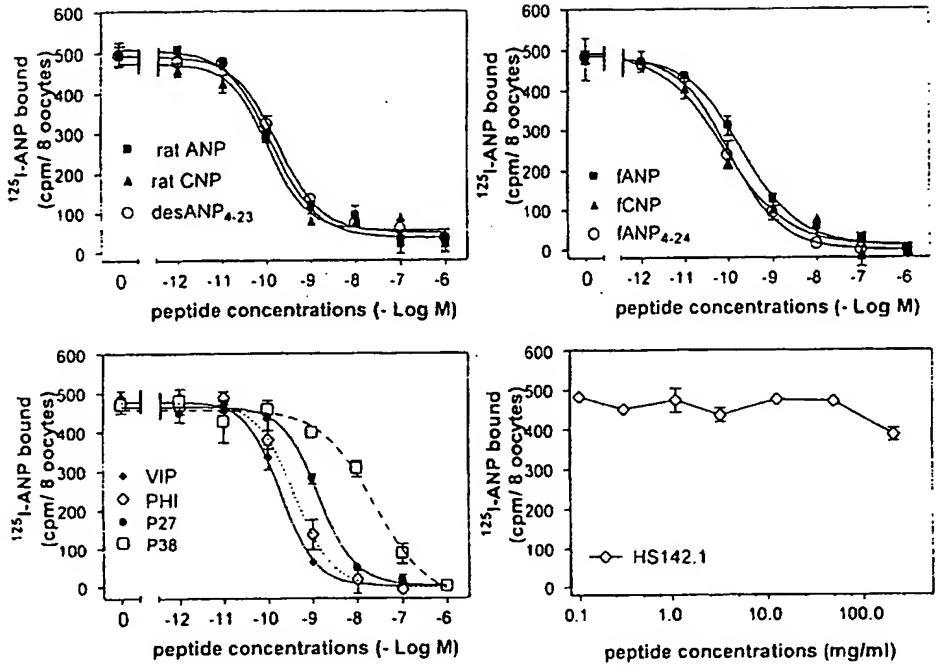

125I-ANP bound (cpm/ 8 oocytes)
rat ANP
rat CNP
desANP4-23
peptide concentrations (- Log M)
fANP
fCNP
fANP4-24
peptide concentrations (- Log M)
VIP
PHI
P27
P38
peptide concentrations (- Log M)
HS142.1
peptide concentrations (mg/ml)

Figure 29

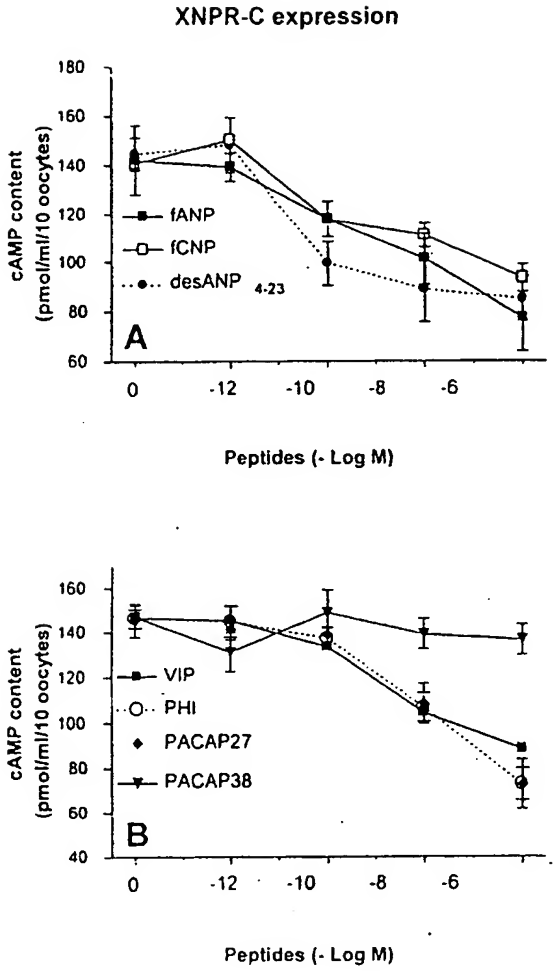

XNPR-C expression
cAMP content (pmol/ml/10 oocytes)
180
160
140
120
100
80
60
fANP
fCNP
desANP 4-23
A
0
-12
-10
-8
-6
Peptides (- Log M)
cAMP content (pmol/ml/10 oocytes)
160
140
120
100
80
60
40
VIP
PHI
PACAP27
PACAP38
B
0
-12
-10
-8
-6
Peptides (- Log M)

Figure 30

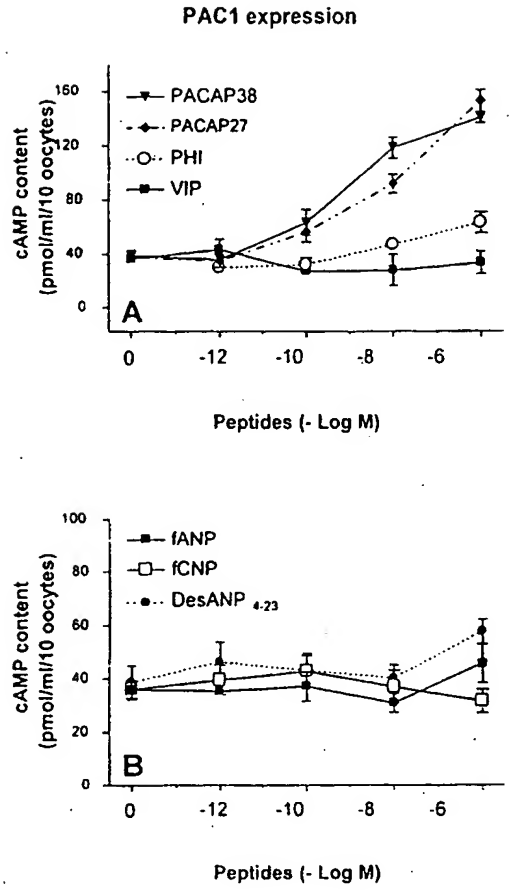

PAC1 expression
cAMP content (pmol/ml/10 oocytes)
PACAP38
PACAP27
PHI
VIP
A
Peptides (- Log M)
fANP
fCNP
DesANP 4-23
B
Peptides (- Log M)

Figure 31

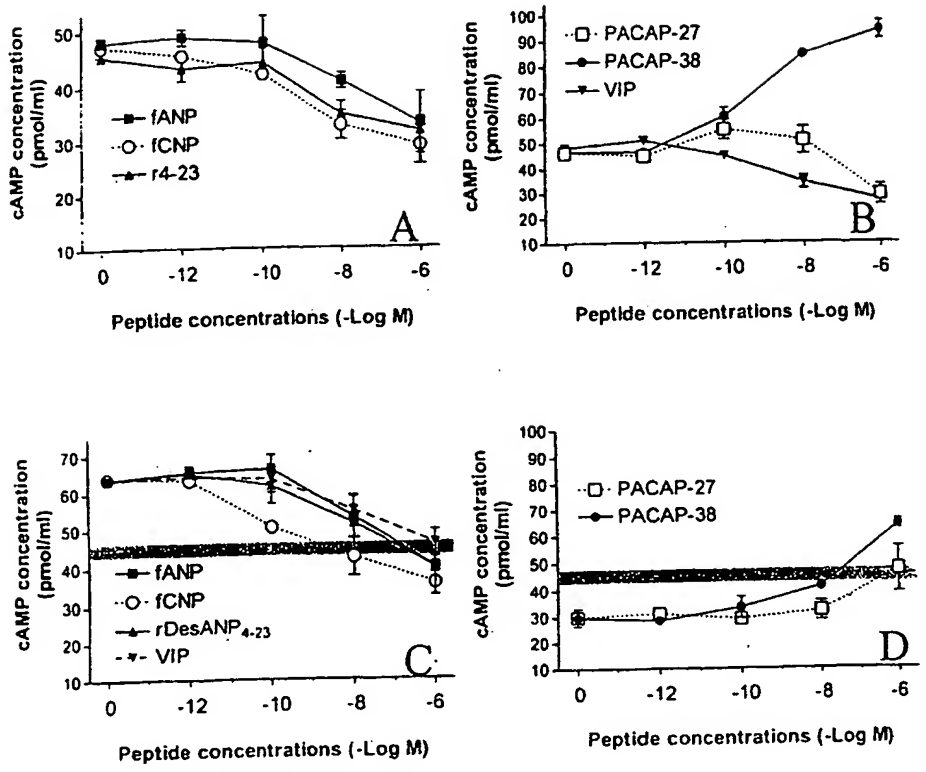

cAMP concentration (pmol/ml)
fANP
fCNP
r4-23
A
Peptide concentrations (-Log M)
PACAP-27
PACAP-38
VIP
B
Peptide concentrations (-Log M)
fANP
fCNP
rDesANP4-23
VIP
C
Peptide concentrations (-Log M)
PACAP-27
PACAP-38
D
Peptide concentrations (-Log M)

Figure 32

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US00/24457

A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) : C12N 5/00, 5/02; A01N 43/04; A61K 31/70; C07H 21/04
US CL : 435/375; 514/44; 536/23.1, 24.5

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
U.S. : 435/375; 514/44; 536/23.1, 24.5

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
Please See Continuation Sheet

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 98/26610 A1 (I.D.M. IMMUNO-DESIGNED MOLECULES) 28 May 1998 (28.05.98), see entire document.	1-5, 8-9, 11-12 , 16-17
A	DREWETT J. G. et al. Neuromodulatory Effects of Atrial Natriuretic Factor Are Independent of Guanylate Cyclase in Adrenergic Neuronal Pheochromocytoma Cells The Journal of Pharmacology and Experimental Therapeutics. 1990, Vol. 255, No. 2, pp. 497-503 see entire document	1-5, 8-9, 11-12 , 16-17

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
15 November 2000 (15.11.2000)	28 NOV 2000
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703)305-3230	Authorized officer JANET EPPS Telephone No. 703-308-8883

Form PCT/ISA/210 (second sheet) (July 1998)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US00/24457

Box I Observations where certain claims were found unsearchable (Continuation of Item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claim Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☒ Claim Nos.: 13-15
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
Claims 13-15 recite specific nucleotide and amino acid sequence information, however Applicants have not provided a searchable sequence listing.

3. ☒ Claim Nos.: 6-7 and 10
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of Item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest
☐ The additional search fees were accompanied by the applicant's protest.
☐ No protest accompanied the payment of additional search fees.